Dissipation and Phytotoxicity of Oil Sands Naphthenic Acids in Wetland Plants

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ABSTRACT

Naphthenic acids (NAs) are toxic organic acid compounds released during the caustic hot-water extraction of crude oil from oil sands in north-eastern Alberta, Canada. NAs subsequently accumulate in the large volume of oil sands process water (OSPW) produced daily by oil sands operations. The complexity of dealing with a mixture of over 200 individual NA compounds, combined with their acute aquatic toxicity and large volume of production has made them an emerging pollutant of concern for western Canada. The following thesis outlines a variety of experiments designed to determine the potential to use wetland plants to enhance the dissipation of NAs from OSPW (phytoremediation).

Investigations were carried out with three native emergent macrophyte species cattail (*Typha latifolia*), common reed (*Phragmites australis* subsp. *americanus*), and hard-stem bulrush (*Scirpus acutus*) to see if they enhanced the dissipation of NAs from a hydroponic system. Dissipation of NAs (at 30 mg L^{-1} and 60 mg L^{-1}) was investigated with both a commercially available NA mixture as well as with a NA mixture extracted from the OSPW. Dissipation of NAs was also investigated under the different ionized forms of NAs (ionized, pH = 7.8; and non-ionized, pH = 5.0) to better elucidate the mechanisms of NA uptake and toxicity in plants. Phytotoxicity of NAs was investigated in hydroponic experiments through fresh weight gain and evapotranspiration was monitored throughout the experiment by water uptake. Commercially available NA mixture was more phytotoxic than oil sands NAs mixture. As well, NAs were found to be more phytotoxic in their non-ionized form therefore indicating that they may be taken up through an 'ion-trap' mechanism. However despite this, no significant dissipation of total NAs was observed from planted hydroponic systems. Nevertheless there was a significant change in the distribution (percent abundance) of individual NA families of certain size. These changes were related to the one- and two-ring NA compounds (Z = -2 and Z = -4). Despite not detecting any dissipation of total NAs from the systems, plants were able to reduce the toxicity of a NA system over 30 days by 45% as determined by *Daphnia magna* acute toxicity bioassays; a 11% greater reduction than unplanted systems.

Studies were also conducted investigating the microbial community inhabiting cattail roots exposed to NAs. It was observed that the rhizosphere community changed with NA exposure, with a general increase in potentially pathogenic bacteria and a decrease in bacteria previously found to be beneficial to plant growth. The observed microbial community change could be an indirect effect of the phytotoxicity experienced by aquatic macrophytes exposed to NAs. Synchrotron-sourced, fourier transform microspectroscopy analysis of root cross sections revealed that there were significant physiological changes to those roots exposed to NAs. These changes were identified as being cell death in the plant root epidermis as well as a change in the chemistry of parenchyma cells in the root pith. It is not known if these changes are a direct effect of NAs to the plant or due to changes of the associated rhizosphere community in the roots or some combination of both these factors.

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ACN – acetonitrile

AMF – arbuscular mycorrhizal fungi

ANOVA – analysis of variances

APS – apparent photosynthesis

ASE – Accelerated Solvent Extraction, (Dionex Corporation)

atm – Atmospheres

BSA – bovine serum albumin

^{o}C – degrees Celcius

C – carbon

CLS – Canadian Light Source Inc.

CFU – colony forming units

CO_3^{-2} – carbonate ion

cm – centimeter

cm^{-1} – wavenumber

CYP 450 – cytochrome P450 phase I enzyme

DE – diatomaceous earth

DGGE – denaturing gradient gel electrophoresis

DIC – dissolved inorganic carbon

DO – dissolved oxygen

DOC – dissolved organic carbon

DNA – deoxyribonucleic acid

EC_{25} – effective concentration (25%)

EC_{50} – effective concentration (median, 50%)

EDTA – ethylenedinitrilotetraacetic acid

ESI – electrospray ionization

Fe – Iron

FT – fine tailings

FTIR – Fourier transform infrared

g – gram

GC-MS – gas chromatography-mass spectrometer

h – hour

HCl – hydrochloric acid

H_2SO_4 – sulfuric acid

IB – ion balance

IC – ion chromatography

ICP – inductively coupled plasma (mass spectrometry)

IR – infrared

K_a – weak acid dissociation constant

K_d – dissociation constant

KBr – potassium bromide

kg – kilogram

K_H – Henry's Law constant

km – kilometers

km^2 – square-kilometer

K_{oc} – organic carbon partitioning coefficient

K_{ow} – octanol to water partitioning coefficient

KOH – potassium hydroxide

K-S – Kolomogorov-Smirnov

L – Liters

LC – liquid chromatography

LC_{50} – concentration that results in 50% lethality in an organism

LOAEL – lowest observed adverse effect level

LOEC – lowest observed effect concentration

LT27A – acrylamide polymer used for consolidating fine tailings

m – meters

M – molar, mol L^{-1}

m^3 – cubic meters

mA – milliamps

MeOH – methanol

MeV – mega electronvolt

MFO – mixed function oxidase

MFT – mature fine tailings

mg – milligram

mg L^{-1} – milligram per Liter

mL – milliliter

MilliQ – deionized and ultra-filtered water

min – minutes

mol - moles

MPP – monopotassium phosphate buffer

MS – mass spectrometry

m/z – mass to charge ratio

n – number of individuals in a sample (number of replicates)

n – a part of the molecular formula for naphthenic acids $C_nH_{2n+Z}O_2$ a family classification indicating the CH_2 group in NA molecular structure

N – normal (or normality)

N_2 – nitrogen (gas)

NA – naphthenic acid

Na^+ – sodium ion

NAA – naphthalene acetic acid

NaOH – sodium hydroxide

NCBI – National Center for Biotechnology Information

ng – nanograms

NH_4OH – ammonium hydroxide

NOAEL – no observed adverse effect level

OSPW – oil sands process water

P – p-value

PAH – polycyclic aromatic hydrocarbon

PCR – polymerase chain reaction

PDI – protein disulphide isomerase

pH – the inverse logarithmic representation of the hydrogen proton $[H^+]$ concentration.

pK_a – the negative decimal logarithm of K_a

PLE – pressurized liquid extraction.

pp – pages

ppb – parts per billion

ppm – parts per million

psi – pounds per square inch

PVPP – polyvinyl polypyrrolidone

RNA – ribonucleic acid

RO – runoff water

ROS – reactive oxygen species

rpm – rotations per minute

RuBisCo – ribulose-1,5-bisphosphate carboxylase

s – seconds

SD – standard deviation

SE – standard error

$(SO_4)^{2-}$ – sulphate ion

SOC – semi-volatile organic compound

SPE – solid phase extraction

spp. – species

TAE – tris-acetate buffer

TE – tris-EDTA buffer

TSA – trypticase soy agar

UPGMA – sdljf

UV_{254} – ultraviolet light at $\lambda = 254$

V – volts

V_P – vapour pressure

Z – naphthenic acid family classification indicating hydrogen deficiency

λ – wavelength

µL – microliter

µm – micrometer

µS cm^{-1} – microsiemens per centimeter (measure of conductivity)

16S rRNA – a subunit in prokaryotes in ribosomal R

1.0 General Introduction

As a group, naphthenic acids (NAs) are classified as organic acid surfactant compounds composed predominately of alkyl-substituted cycloaliphatic carboxylic acids and to a lesser extent acyclic aliphatic acids. The general chemical formula for naphthenic acids is $C_nH_{2n+z}O_2$ where n is equal to the number of carbon atoms, and Z is equal to either a zero or negative even number and represents the number of hydrogen atoms lost as a result of the structures becoming more compact. For example a NA with a linear or branched hydrocarbon chain is equal to Z = 0, with one ring Z = -2, with two rings Z = -4, and with three rings Z = -6. The carboxylic acid group is most often found attached to a side chain rather bonded to the cycloaliphatic ring in the NAs with $Z \leq -2$. (Fan, 1991; Hsu et al., 2000; Barrow et al., 2003; Headley et al., 2007) (Figure 1.1).

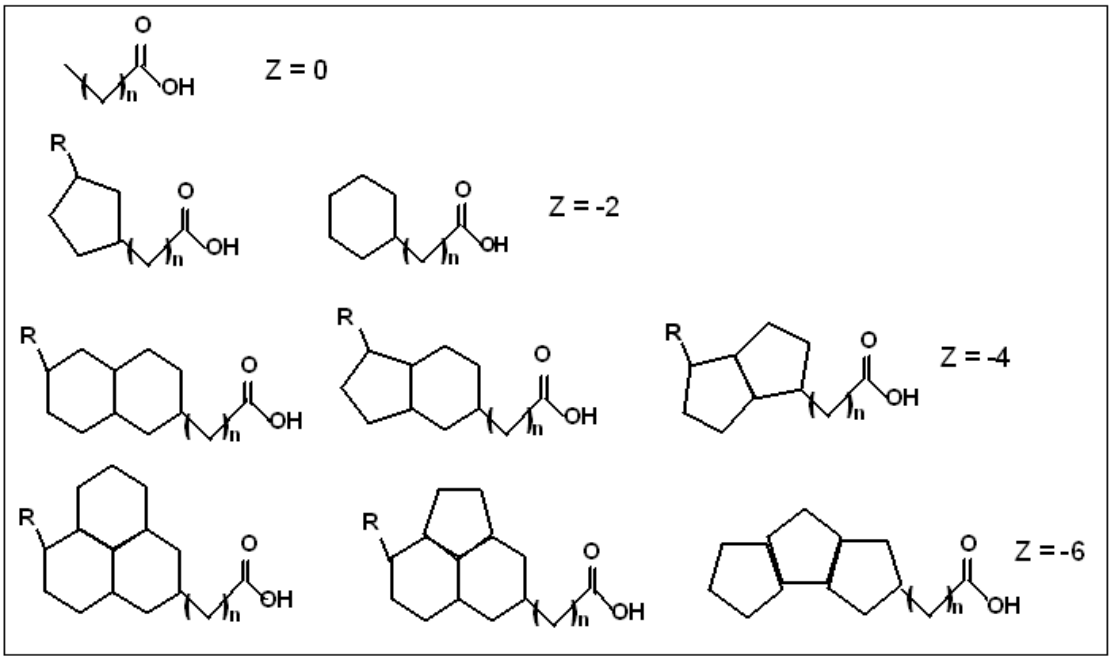

Figure 1.1: General molecular structures of naphthenic acids grouped according to Z family (Z = 0 to Z = -6) (adapted after Headley et al., 2007).

Naphthenic acids are a complex mixture of compounds found naturally in petroleum deposits (Fan, 1991) and are in particular an issue with the mining and processing of oil sands for crude oil. Their concern with oil sands mining and processing is that the caustic hot water extraction process (85^{o}C temperature, NaOH, pH = 8.5) used to separate crude oil from the bitumen solubilizes NAs and concentrates them in the resulting process water (Dorn et al., 1992; Chalaturnyk et al., 2002). Unrefined bitumen from the Athabasca oil sand deposit has ~2% concentration of carboxylic acids identified as NAs. Of this 2% fraction, nearly 90% of it is made up of Z = -6 (tricycloalkane) NAs (Headley et al., 2007).

Alberta's oil sands are made up of three main deposits: Athabasca, Cold Lake, and Peace River; covering a surface area of over 77,000 km^2 (Chalaturnyk et al., 2002). Combined, these oil sands deposits are one the worlds largest, accounting for approximately 85% of the world's total in place* bitumen (Greene et al., 2007) and estimated to contain up to 1.7 trillion barrels (279 billion m^3) of crude bitumen in place. Only ~11% of this amount or 174 billion barrels (27.7 billion m^3) is considered to be recoverable under current technology and economic conditions (Söderbergh et al., 2007). Mining and processing oil from the bitumen contained in Alberta's oil sands deposits has been occurring at the Athabasca deposit in Fort McMurray, Alberta, by two main commercial companies Suncor Energy Inc. since 1967 and Syncrude Canada Ltd. since 1978 (Mikula et al., 1996). As conventional sources of petroleum deplete, demand for oil increases, and interest in foreign oil reserves wanes, the switch to mining unconventional oil deposits like Alberta's oil sands is only expected to increase (Greene et al., 2006; Söderbergh et al., 2007). Moritis (2004) reports that production is expected to increase to over 95% of its 920,000 barrels (146,000 m^3) per day in 2003 to over 1.8 million barrels (286,000 m^3) per day by 2010. This increased production has come from both improvements in technologies for bitumen recovery as well as through the proposed development of an additional 7 major oil sands mining projects from the existing five oil sands projects in place as of 2003 (Moritis, 2004).

* In place refers to the total amount of oil in an oil reservoir. However the total amount of oil in place in a reservoir may not be completely accessible for extraction due to limitations in extraction technology.

Unfortunately, extracting crude oil from the bitumen found within oil sands requires inputs of large volumes of water; a result of the caustic hot water extraction process. Approximately 12 m^3 of water is required to produce crude oil from every cubic meter of mined oil sand. Aproximately 70% of this amount of water is recycled leaving a net production of average of 4 m^3 of liquid waste (Mikula et al., 2008). Due to the unknown effect that these liquid tailings (herein referred to as oil sands process water; OSPW) will have on the surrounding environment, companies mining the oil sands are held to a zero discharge policy and no OSPW is knowingly released into the environment outside their lease areas. As a result of this, the great volumes of OSPW produced are stored in large tailings ponds on mine sites. If production reaches the forecasted production rate of 1.8 million barrels (286,000 m^3) per day by 2010 then daily water inputs required for extraction at this time will be at least 5.4 million barrels (858,000 m^3) and daily production of OSPW will be at least 7.2 million barrels (1.1 million m^3) per day. When considering the ramifications of future expansion and development of oil sands operations in Alberta, it becomes apparent that effects will manifest not only as impacts on the local hydrology (Schindler and Donahue, 2006) but also as purely a waste management issue for the oil sands mining companies for the storage of OSPW (Mikula et al., 1996).

Oil sands process water is affected by a number of water quality issues including turbidity due to the presence of clay fines, high concentrations of dissolved ions (primarily Na^+ and SO_4^{2-}, heavy metals, and toxic organics such as polycyclic aromatic hydrocarbons (PAHs) and NAs) (Mikula et al., 1996; Crowe et al., 2001). Of these, NAs are of most concern in the OSPW because they are highly water soluble and therefore have the potential to enter into aquatic environments surrounding oil sands operations (Bishay and Nix, 1996) (Section 1.2). In addition to their water solubility, NAs are also of toxicological concern in the environment because they are acutely toxic to a variety of fish species and aquatic invertebrates with LC_{50} ranging from 4 – 78 mg L^{-1} (Dokholyan and Magomedov, 1983; Dorn et al., 1992; Verbeek et al., 1994; Herman et al., 1994) (Section 1.3).

While there are methods currently available to clarify the OSPW of clays and to remove dissolved ions, removal of recalcitrant organic chemicals like NAs has proven to be a challenge (Nix and Martin, 1992; Crowe et al., 2001). Biodegradation of NAs by microorganisms has been investigated by several groups with various levels of success (Nix and Martin, 1992; Herman et al., 1994; Lai et al., 1996; Clemente and Fedorak, 2005; Quagraine et al., 2005; Biryukova et al., 2007) (Section 1.8). However investigations on the factors driving the ability of plants to remediate NAs (phytoremediation) have received little attention to date.

Phytoremediation is the use of plants and their associated microorganisms for the *in situ* treatment of contaminants (Siciliano and Germida, 1998; Arthur et al., 2005). Phytoremediation has been used successfully to treat contaminated soils (Germida et al., 2002). In addition, constructed wetlands with submergent and emergent macrophytes have been used to treat contaminated water (Gschlöβl et al., 1998; Murray-Gulde et al., 2003; Kanagy et al., 2008) (Section 1.7). Plant roots provide a large surface area optimizing the opportunity for interaction of environmental contaminants with the root surface. Additionally, plants produce root exudates comprised of a variety of chemicals such as amino acids, sugars, and vitamins which are beneficial to the many bacteria and mycorrhizal fungi. These exudates are produced to attract and promote the growth of microorganisms inhabiting the rhizosphere that in turn provide the plant with better uptake of nutrients as well as even some protection from pathogenic bacteria (Dakora and Phillips, 2002).

Previous research confirmed the ability of indigenous aerobic microbial communities found in oil sands tailings ponds to biodegrade NAs (Herman et al., 1994; Clemente and Fedorak, 2005; Biryukova et al., 2007). It is anticipated that the combination of wetland plants with NA-degrading bacteria will form a symbiotic relationship and together be an effective means for mitigating NAs present in OSPW. Nevertheless, the extent of such relationship has yet to be confirmed in wetland plants exposed to OSPW. Nix and Martin's (1992) research on the effect of tailings pond water on the algae *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) have shown that tailings pond water can be stimulatory. In addition, work with higher Aquatic macrophytes such as cattails (*Typha latifolia*) has displayed signs that wetland

plants can adapt to tailings pond water (Crowe et al., 2001). Elevated concentrations of ions such as Na^+ and SO_4^{2-} have been identified to be the greatest concern in plants exposed to OSPW, resulting in osmotic stress (Renault et al., 1998; Renault, 2005). Potential for uptake and metabolism of NAs by wetland plants is further supported by Doucette and coworkers (2005) who found that other water soluble petroleum production chemicals such as di-isopropanaolamine and sulfolane were readily taken up by cattail and sequestered from the environment.

1.1 Physical and Chemical Properties of Naphthenic Acids

Naphthenic acids are considered to be surfactants because they have both a hydrophilic end (carboxyl group) and a hydrophobic end (non-polar aliphatic end). They have a water solubility that ranges between 0.070 mg mL^{-1} to 5.04 mg mL^{-1} at 25°C between the pHs of 0.91 to 9.16 respectively (Table 1.1). At 3°C, the range drops down

Table 1.1: Physical and chemical properties of naphthenic acids (Baker Chemical commercial mixture) (adapted from CEATAG, 1998).

Property	Value
Water Solubility at 25°C	@ pH = 0.91: 0.070 mg mL^{-1} @ pH = 9.16: 5.04 mg mL^{-1}
Water Solubility at 3°C	@ pH = 0.91: 0.060 mg mL^{-1} @ pH = 9.16: 4.52 mg mL^{-1}
Vapor Pressure (V_p)	2.35 x 10^{-6} atm
Henry's Law Constant (K_H) @ solubility = 70 mg L^{-1} and MW = 255 g	mol^{-1}: 8.56 x 10^{-6} atm·m^3·mol^{-1}
Log octanol to water partitioning coefficient ($logK_{ow}$)	@ pH 1.07: 4.3 @ pH 7.1: 2.38 @ pH 10: 2.08
Disassociation constant (pK_a) at 25°C	5.2

to between 0.060 mg mL^{-1} and 4.52 mg mL^{-1} respectively. Solubility of organic acids like NAs in water is reduced in aqueous salt solutions (Xie et al., 1997). This "salting-out effect" is important to consider for OSPW because OSPW contains high levels of salts (Mikula et al., 1996; see section 1.7.1). The vapor pressure (V_p) of these chemicals is estimated to be around 2.35 x 10^{-6} atm with an estimated Henry's Law Constant (K_H) of 8.56 x 10^{-6} atm·m^3·mol^{-1} at a solubility of 70 mg L^{-1} and a molecular weight of 255 g mol^{-1}. The log octanol to water partitioning coefficient ($logK_{ow}$), a measure of the propensity of these chemicals to bioaccumulate in lipids, is 4.3 at pH 1.07, 2.38 at pH 7.1, and 2.08 at pH 10. The disassociation constant (pK_a) of these compounds at 25°C is 5.2 (CEATAG, 1998). With regards to their susceptibility to photodegrade, McMartin et al. (2004) found that NA photodegradation in natural and artificial sunlight was limited in natural river water. The authors also concluded that some NAs are more susceptible than others and that UV_{254} radiation was able to accelerate the photodegradation rate of some individual NAs (McMartin et al., 2004).

Based on the mass spectra displayed in Figure 2.6 and a concentration of 60 mg L^{-1}, oil sands NA mixture is estimated to have a range of molarity between 4.0 x 10^{-4} mol L^{-1} to 1.4 x 10^{-4} mol L^{-1}. The mode (or most common occurring molarity) was found to be 2.7 x 10^{-4} mol L^{1} in oil sands NAs. In contrast a commercially available NA mixture, Fluka, available from Sigma-Aldrich Ltd. had a range of molarity in a 60 mg L^{-1} solution estimated to be between 3.8 x 10^{-4} mol L^{-1} and 1.9 x 10^{-4} mol L^{-1} (Figure 2.6). The most common molarity in the Fluka mixture was 2.0 x 10^{-4} mol L^{-1}.

1.2 Fate of Naphthenic Acids in the Environment

The OSPW resulting from mining bitumen have an alkaline pH ranging between 7.7 and 8.5 depending on the mining company and location (e.g. surface water vs. fine tails) (Mikula et al., 1996). Because the mean pK_a of NAs is 5.2 (Table 1.1), most NAs are ionized and soluble in the aqueous phase in the alkaline OSPW. The subsequent solubility of NAs in alkaline OSPW is what aids in their movement in the aquatic

environment within which they are released. Tailings ponds containing OSPW have NA concentrations ranging from 1 mg L^{-1} to as high as 120 mg L^{-1} (CEATAG, 1998; Headley and McMartin, 2004; Clemente and Fedorak, 2005), which is well above the no observed adverse effect level (NOAEL) for several species of fish (Section 1.3.1). Bishay and Nix (1996), Bendell-Young et al. (2000) and Crowe et al. (2001) all report seepage of OSPW from tailings ponds into wetlands within the oil sand leases, despite the zero discharge policy applied to the oil sands industry in Alberta. Although companies make efforts to collect seepage water and pump it back into tailings ponds, seepage is a concern considering the fact that two of the largest oil sands mining operations (Syncrude Canada Ltd. and Suncor Energy Inc.) and their associated tailings ponds are located beside the Athabasca River.

Studies on the adsorptive properties of model NA compounds (cis and trans isomers of 4-methylcyclohexaneacetic acid; and cis and trans isomers of 4-methylcyclohexanecarboxylic acid) found that they have a low K_d (0.10 – 0.22 mL g^{-1}) and are therefore not likely to partition to soils and sediments (Peng et al., 2002). Headley and McMartin (2004) hypothesized that their low K_d together and relatively high water solubility; this would ensure that NAs are free in aquatic environments and potentially bioavailable for either microbial degradation or uptake by plants. However, despite the solubility and low K_d observed in model NA compounds, Janfanda et al. (2006) found evidence of selective sorption of oil sands NA mixture and that soils with higher organic content would have greater attenuation of oil sands NAs. They found K_d ranging between 1.3 mL g^{-1} in MilliQ water to 17.8 mL g^{-1} in synthetic ground water (Janfanda et al., 2006). The discrepancies between these two studies likely stem from the fact that ~90% of NAs in the oil sands crude oil are multicyclic carboxylic acids ($Z > -2$) (Section 1.1) whereas model compounds used by Peng et al. (2002) were monocyclic carboxylic acids ($Z = -2$).

1.3 Toxicity of Naphthenic Acids

Naphthenic acids have been identified as one of the main toxic components of concern in OSPW (Dorn et al., 1992). In their ionized, naphthenate salt form (pH > 6), NAs are considered to be lower in toxicity because they are polarized and not likely to pass through biological membranes. When in their non-ionized form (pH < 6) NAs are found to be more toxic. This is most likely because these compounds are lipid soluble in this state and therefore more capable of dissolving into the lipid membrane of biological tissue and eliciting toxicity. Dorn et al. (1992) report that decreasing the pH of an effluent from a California oil refinery known to contain NAs from 7.8 to 6.6 caused the three spine stickleback fish (*Gasterosteus aculeatus*) survival rate to drop from 100 to 0 percent. Another consideration with NA toxicity is the source of the mixture. The toxicity of the NA extract depends on source (e.g. commercial vs. oil sands extract). Commercial NA extracts, which have been used in many previous toxicity studies to predict the fate and effects of oil sands NAs, have been found to be more toxic than oil sands NAs (Scott et al., 2005; Armstrong et al., 2008). This discrepancy has been attributed to the lower molecular weight compounds present in commercial NA mixtures compared to the higher molecular weight compounds found within the oil sands NA extract. The lower molecular weight components are likely more capable of interacting with biological tissue (Clemente and Fedorak, 2005).

1.3.1 Fish

Naphthenic acids have been found to be acutely toxic to a variety of fish species with LC_{50} values ranging between 4 to 78 mg L^{-1} (Dokholyan and Magomedov, 1983; Dorn et al., 1992; Verbeek et al., 1994; Herman et al., 1994). These LC_{50} concentrations are also dependent on the water chemistry for which the NAs are dissolved in as well as the source of NAs (Headley and McMartin, 2004). van den Heuvel et al. (1999) found that yellow perch (*Perca flavescens*) exposed to waters associated with oil sands reclamation do not compromise the short-term physiological status of exposed fish. In these experiments the authors tested a water-capping reclamation strategy under

investigation. There was no direct evidence to suggest that oil sands related compounds were responsible for reduced survival, condition gonadal growth in yellow perch however as the authors stated physiological indices in adult fish are not the most sensitive indicators of toxicant stress. In addition, impacts may be masked by compensatory responses at both the individual and population level. In these studies NAs were reported to be in their naphthenate form and all under 9.1 mg L^{-1}. Both Siwik et al. (2000) and Nero et al. (2006) conducted similar studies with different OSPW under field conditions with fathead minnow (*Pimephales promelas*) and yellow perch, respectively, and drew similar conclusions to van den Heuvel et al. (1999). While Nero et al. (2006) found sub-lethal gill and liver alterations in yellow perch (*Perca flavescens*) and caged goldfish (*Carassius auratus*) these effects could be attributed to a combination of elevated NAs (1.4 – 24 mg L^{-1}), PAHs, and salinity found in the OSPW. Siwik et al. (2000) found increases in larval fish length after 7 days exposure to different OSPW amended wetlands but not at 28 or 56 days. The increased larval length observed after seven days was thought to be an example of a hormetic effect by the treatment waters in the fish. Concentrations of NAs found in the treatment waters used by Siwik and coauthors (2000) ranged between 3.3 and 59.4 mg L^{-1}.

1.3.2 Aquatic invertebrates

Minimal research has been conducted on the specific effects of oil sands NAs in aquatic invertebrates. All invertebrate studies to date have been conducted with OSPW which contains a mixture of potentially toxic components including NAs. CEATAG (1998) reviewed the available studies on invertebrate NA toxicity and only found one report that LC_{50} of OSPW in the freshwater crustacean *Daphnia magna* ranged from 76 – 98% and if the concentration of the tailings pond water was assumed to be 80 mg L^{-1} this would equate to an LC_{50} of 61-78 mg L^{-1}. In their study characterizing a wetland receiving effluent from an oil sands operation Bendell-Young et al. (2000) found that impacted wetlands had an aquatic invertebrate community which was less similar and less diverse than reference wetlands as indicated by significantly higher chironomid (Chironomide spp.) density and biomass in impacted wetlands. In addition, within the chironomid populations found within the OSPW exposed wetlands there was a greater

percentage (~8%) of deformities observed in the mentum. Morphological changes of the mentum (projecting structure near the mouth) are frequently used to gauge environmental disturbance (Bendell-Young et al., 2000). In all these exposed wetlands, water pH was between 7.7 - 8.1 and therefore NAs would have been in their predominantly ionized form. Naphthenic acid concentrations are not reported in this study but likely range between 3.0 to 70 mg L^{-1} as reported by Crowe et al. (2002) who used some of the same sites for their study.

1.3.3 Mammals

Rogers and coworkers (2002b) determined that NAs extracted from the Athabasca oil sands resulted in significant changes in physical, clinical and pathological condition with both acute (300 mg kg^{-1}) and subchronic (60 mg kg^{-1} day^{-1}) exposure in Wistar rats. The authors of this study concluded that their observations pointed to the liver as the target organ of NAs in the mammalian system (Rogers et al., 2002b). The authors reported elevated liver weights in rats and suggested that this may be a result of cytochrome P450 (the enzymes responsible for biotransforming xenobiotics) induction. To date, there have been no further investigations on the ability of cytochrome P450 enzymes to biotransform NAs into less toxic forms in mammals as well as in other organisms (fish, plants, invertebrates, etc.).

1.3.4 Plants and algae

Organic acid accumulation in wetland sediments as a result of microbial metabolism under oxygen deficiency in sediment has been previously found to be phytotoxic to common reed (*Phragmites australis*) (Armstrong et al., 1996; Čížková et al., 1999). Some of the reported effects to common reed exposed to acetic acid included: restricted adventitious root, lateral root and bud growth, death of root apices and buds, and abmormal lignification and callus formation in roots and rhizomes causing blockages in the xylem and phloem of the vascular system (Armstrong et al., 1996). Jackson and Taylor (1970) report that barley roots exposed to organic acids in their undissociated form alters root membranes to become more permeable to ion influx and efflux.

In mesocosm experiments Bendell-Young et al. (2000) noted that OSPW resulted in significantly greater photosynthetic rates in cattails (*Typha latifolia*) than their unexposed counterparts. Although this observation was made, it was unclear at the time what the finding meant with regards to the long term survival of the plant. To determine the physiological effect of OSPW, Crowe and coworkers (2001) exposed cattail and alsike clover (*Trifolium hybridum*) to OSPW, and monitored apparent photosynthesis (CO_2 uptake, APS) as well as steady-state ribulose-1,5-bisphosphate carboxylase (RuBisCo) levels. RuBisCo is the enzyme in photosynthesizing cells responsible for 'fixing' inorganic carbon in the form of carbon dioxide into sugars. A decrease in the amount of this protein would indicate interference in the plants' ability to produce energy. Two stress proteins that accumulate in osmotically stressed plants, dehydrin and protein disulfide isomerase (PDI), were also monitored in the plants to determine the contribution of osmotic stress from the OSPW to plant toxicity. In plants growing in wetlands most heavily influenced by OSPW there was a significantly greater APS rate compared to plants sampled from a reference wetland site. However, the levels of RuBisCo were similar in plants from all sites. Plants subject to an osmotic stress are expected to exhibit a decreased rate of photosynthesis due to the a) reduction of the CO_2 supply due to closure of the stomates and 2) non-stomatal events mediated by reduced permeability to CO_2 in the leaf, effects on chloroplast metabolism and altered source-sink relations within the plant. Therefore the high APS rates observed by Crowe et al. (2001) were unexpected although consistent with research by Bendell-Young et al. (2000). Growth was not compromised even though some wetlands had 70 mg L^{-1} NAs. Crowe and coworkers (2001) concluded that the NAs were not responsible for the elevated of APS in wetland plants exposed to oil sands effluent because APS was high in wetlands with both high and low NA concentrations. Overall, the authors concluded that cattail and clover are well-adapted to growth in the OSPW but that further studies were warranted to determine their long term ability to survive in these OSPW affected systems.

Kamaluddin and Zwiazek (2002) investigated the effect of a commercial NA mixture (Acros, Geel, Belgium) on root water transport, gas exchange and leaf growth in trembling aspen (*Populus tremuloides*) seedlings. They hypothesized that because of their surfactant properties, NAs adversely affected plants by interfering with root water

flow through adhesion to the root surface. In the 150 mg L^{-1} and 300 mg L^{-1} treatments root xylem exudates collected from roots within a pressure chamber were found to contain 15 mg L^{-1} and 21 mg L^{-1}, respectively, using FTIR spectroscopic analysis of the methylene chloride extracts. However, these findings should be used with caution as the treatment concentrations are several times higher than what plants would be exposed to in the OSPW, the authors used a pressure chamber on the roots, which can force compounds into the plant tissue that would not move into the tissue otherwise, and the FTIR spectroscopy method may account for other endogenous carboxylic acid compounds found within the plant tissue (pers. comm. J. Zwiazek, 2005). Furthermore it is likely not valid to apply the surfactant properties of NAs as the cause of phytotoxicity when in the OSPW NAs are found as naphthenate salts. Finally, the NA extract used in this study was a commercial extract with a mixture of lower molecular weight compounds, as well, these results have yet to be replicated for plant tissue.

In the same study, root hydraulic conductance and stomatal conductance were significantly decreased by about 43% and 40% of their control counterparts respectively by all NA treatments. All treatment concentrations of NAs significantly reduced the photosynthetic rate of the seedling and the leaf chlorophyll concentrations. Photosynthetic rate decreased with increasing NA concentration in the root medium. At the highest NA concentration (300 mg L^{-1}) the photosynthetic rate was 40% of the control. Leaf expansion ratios were greater than 1 for control aspen seedlings. However, for seedlings in all the NA treatments the leaf expansion ratios were reduced by at least 50% for the lowest NA treatment. Root water flow rates decreased rapidly in NA treated aspen seedling roots compared to control roots. Reduced root water flow rates were observed within 30 minutes of addition of NAs to the root medium and rates were reduced to 52-57% of pre-treatment flows 1.5 hours post NA treatment. Finally, NAs were also found to significantly reduce the O_2 uptake rates of the seedling root systems. Two hours post treatment with NAs, root O_2 uptake was reduced to 70% of the pretreatment rate in all NA treatments (Kamaluddin and Zwiazek, 2002).

Naphthenic acids are expected to aggravate the effects of the saline conditions of OSPW on plants. The inhibition of root water flow by NAs could possibly be a result of their surfactant properties and also result in the decreased stomatal conductance.

However since Kamaluddin and Zwiazek (2002) also found NAs in the root xylem, they concluded that NAs could also be affecting the root water flow by interfering with plant metabolism. Water channels known as aquaporins are responsible for the majority of transmembrane root water transport (Tyerman et al., 1999). Therefore, reductions of root water uptake could be a result of changes in the activity of the water channels by NAs. As a result, Kamaluddin and Zwiazek (2002) concluded that that the reduced activity of root water channels caused by metabolic inhibition (indicated by decreased O_2 uptake by roots) may account for the decreased root water flow. The metabolic effect of NAs may vary depending on plant species and NA treatment (Kamaluddin and Zwiazek, 2002).

Stimulation of plant metabolism by some sodium naphthenates has been found in experiments with foliar application in *Phaseolus vulgaris* (Wort et al., 1973). However, further study found that it was a particular compound in the NA mixture, cyclohexanecarboxylic acid, that was causing the enhanced metabolism. Nix and Martin (1992) also found that OSPW high in NAs caused enhanced growth of the fresh water green algae *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*) but this result has yet to be repeated (see Appendix 1). There is very little information available in the literature on the effect of NAs on other freshwater algae. However for the diatom *Nitzschia linearis* a 96 hour LC_{50} was determined to be 43.1 mg L^{-1} in a naphthenic acid solution (CEATAG, 1998). In their evaluation on the effects of OSPW on phytoplankton communities in mesocosms, Leung et al. (2003) found that biomass appeared to be resilient to the effects of non-ionized NAs but the patterns within the community composition suggested thresholds for ecological effects intermediate to the LOEC (7.5 mg L^{-1}) and EC_{50} (19.2 mg L^{-1}) reported for *P. subcapitata*. Hayes (2005) studied the effects of NA and elevated conductivity in natural phytoplankton communities and established NA (24-50 mg L^{-1}) and conductivity (1000 $\mu S\ cm^{-1}$) thresholds for established aquatic systems.

The synthetic auxin, naphthalene acetic acid (NAA), has a similar structure to NAs in the Z = -4 family so the hypothesis of stimulation of plant cells by NAs has some validity (Figure 1.2). Auxins are a group of plant hormones which stimulate cell elongation and also influence numerous other developmental responses (Hopkins, 1999). Although NAA is has an aromatic ring structure and NAs are aliphatic there is potential

that NAs operate through the same receptors as the auxin NAA. However, the study by Kamaluddin and Zwiazek (2002) showed inhibition in response to NAs exposure. This highlights the point raised by Crowe et al. (2001) that because isolation and identification of every individual NA compound present in the NA mixtures found at the oil sands is difficult, information on the specific mode of toxicity of these compounds is lacking. Additionally, if NAs do indeed act through a hormone receptor they could be stimulatory at low concentrations and cause toxicosis and growth reduction at high concentrations.

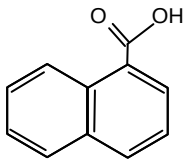

Figure 1.2: Molecular structure of the synthetic auxin naphthalene acetic acid.

Interference by other compounds makes current low resolution mass spectrometry methods for the analysis of NAs in plant extracts unreliable; however, NAs can be analyzed in the xylem sap (Kamaluddin and Zwiazek, 2002), which suggest that they could enter the leaf tissues and affect leaf membrane integrity. In conflict with this finding, the same study found that NA treatments did not alter electrolyte leakage in leaf tissues (Kamaluddin and Zwiazek, 2002). It is not known if the large decreases in leaf chlorophyll and photosynthesis were affected directly or indirectly in root absorbed NAs. The decrease in photosynthesis could be attributed to the decrease in chlorophyll and leaf stomatal conductance. There could be a reduced uptake of essential mineral nutrients due to the surfactant behavior of NAs, which could adversely affect the formation of chlorophyll and photosynthesis processes.

1.4 Analytical Methodologies for Naphthenic Acids

Naphthenic acids are not easily analyzed by conventional low resolution mass spectrometry analytical techniques. Naphthenic acids have a complex mixture of individual compounds with varying molecular weights and structures. As a result of this complex mixture, their polarity and non-volatility increases with molecular weight. Gas chromatography (GC) resolution into individual peaks is difficult; even when the NAs are derivatized with a methyl group to make the compounds volatile enough to move through the GC column better (CEATAG, 1998; Morales et al., 1993; McMartin and Headley, 2004). Clemente and Fedorak (2005) report that despite the advances in analytical methodologies the elucidation of the composition of NA mixtures has not been achieved to date. Analysis and quantification of NAs is usually desired within complex matrices such as nutrient medium, tissue, and sediment. Solid-phase extraction (SPE) or pressurized liquid extraction (PLE) is generally required for sample clean up (Carabias-Martínez et al., 2005). These pretreatment steps are often developed in tandem with analytical methods to achieve results with the highest signal to noise ratio and lowest detection limits possible. In the following two sub-sections, further details will be provided outlining the analytical technique used in the present research as well as the pretreatment steps used prior to analysis.

1.4.1 Negative ion electrospray mass spectrometry

In the present study, identification and quantification of NAs was achieved using negative ion electrospray ionization low resolution mass spectrometry (ESI-MS). Electrospray ionization is a 'soft' chemical ionization technique to prevent the complete fragmentation of parent molecular ions. With negative ion electrospray, a loss of a proton from the molecular ion [M] can leave the $[M-H]^-$; an intense negatively charged ion. For mixtures containing carboxylate groups such as NAs, these ions are often the only ones generated and they can then be detected using mass spectrometry with great sensitivity. In the present method, described first by Headley et al. (2002) for NA

analysis in aqueous samples, the electrospray process uses N_2 gas as a buffer gas and analytes in an organic solvent (50:50 acetonitrile:water + 0.1% NH_4OH). The quantification of NAs occurs by integrating the area under the full scan spectrum and compared to a five point calibration curve. The key attractions to this method are: (1) there is no derivatization of analytes as required for GC-MS and (2) water samples can be extracted directly with no extraction steps prior to instrumental analyses.

1.4.2 Extraction and analysis in tissue matrices

For the following research, the extraction of NAs is mainly of interest in two different matrices: hydroponic medium (water + nutrient salts) and plant tissue. In general, the extraction of compounds that contain the carboxylic acid functional group (like NAs) from non-acidic compounds takes advantage of their solubility in an aqueous base. Once separated, the acid can then be regenerated through acidification of the aqueous solution (Morrison and Boyd, 1976). These properties have been taken advantage of for the liquid-liquid extraction and production of oil sands NA extract from OSPW (Rogers et al., 2002a; Janfada et al., 2006). Jones et al. (2001) and Frank et al. (2006) both developed methods to separate naphthenic acids from oil and OSPW respectively using anion exchange columns. A different approach to separate and concentrate NAs from aqueous matrix for clean up prior to analysis was developed by Headley et al. (2002) whereby NAs are acidified into their neutral form and extracted from the aqueous matrix in a solid phase extraction column.

For solid matrices like plant tissue separation and isolation of NAs becomes more complicated because there are usually many endogenous carboxylic acid compounds in plant tissue. Kamaluddin and Zwiazek (2002) analyzed NAs in plant tissue by using a pressure chamber around plant roots to collect xylem sap directly from the cut stem. The xylem sap is already in an aqueous matrix ready for analysis after a methlyene chloride liquid-liquid extraction step. Young et al. (2007) reported a method for NA extraction and analysis from fish tissue. They adapted a method originally developed for the analysis of free fatty acids in fish tissue. Essentially the method involves a solvent extraction using 2:1 chloroform:methanol mixture homogenized with the tissue, acidified,

and then the polar fraction further separated from the tissue. The final clean up step involved the use of an anion exchange column originally developed by Jones et al. (2001) mentioned previously. The methods developed by Young et al. (2007) for fish tissue are currently being tested for use with plant tissue (pers. com. J. Headley, 2008). Further developments into the analysis and extraction of NAs in plant tissue are discussed in Section 5.

1.5 Plant Uptake of Organic Chemicals

Harborne (1984) states that one of the unique features of plant metabolism (when compared to animals and micro-organisms) is their ability to accumulate organic acids in the cell vacuole to high levels. This characteristic is often a factor of importance in intermediary metabolism and plant respiration. It is therefore feasible that there are transporters in place in the vacuole cell wall that can take up NAs as well as endogenous carboxylic acids. Several weak acid herbicides have been found to be taken up in crop plants and in these cases they were found to have increased translocation as the pH of the solution decreased due to reduced ionization of the weak acid (Briggs et al., 1987; Rigitano et al., 1987). The uptake of weak acids is hypothesized to occur via an ion-trap mechanism whereby weak acids enter into plants as a non-ionized neutral compound, are ionized upon entering the cell, and become 'trapped' within the cell (Rigitano et al., 1987).

Riederer (2005) states that pH-dependent dissociation and protonation of xenobiotics within plants is one of the most important factors dictating mobility within the plant. Plant metabolism by chemical modifications such as conjugation or oxidation may also play a role in the eventual fate of xenobiotics in plant tissue (Section 1.6). The xylem is capable of long distance transport of water and small polar solutes (inorganic and organic). Compounds that are transported in the xylem eventually get deposited in the areas of greatest transpiration, which includes the leaf margins and the leaf tip. The requirements for xylem translocation are somewhat contradictory in that the compound must be lipid soluble enough to move into plant cells symplastically to pass through the

endodermis and then be sufficiently hydrophilic to avoid partitioning into lipoid materials. For phloem transport, assimilates are actively pumped into the phloem at the source region, which causes a decrease of water potential resulting in an influx of water. Meanwhile at the sink region, solutes are actively exported from the phloem and water follows osmotically from the phloem. The most phloem-mobile xenobiotics are expected to be weak acids. Weak acids, like NAs, which are non-ionized within the apoplast and xylem (~pH = 5.5) would become trapped in the more basic phloem (~pH = 8) (Riederer, 2005).

1.5.1 Uptake of naphthenic acids by plants

For NAs, because of analytical challenges with their detection in plant tissue, knowledge of uptake from the environment is limited. The only evidence of NA uptake to date was provided by Kamaluddin and Zwiazek (2002) who were able to extract xylem sap from aspen seedlings (*Populus tremuloides*) grown hydroponically in ½ strength Hogland's nutrient media dosed with 150 mg L^{-1} and 300 mg L^{-1} of NAs. Xylem sap was collected and analyzed to contain 15 mg L^{-1} and 21 mg L^{-1} for the 150 and 300 mg L^{-1} NA treatments respectively. However the NA doses used in this study were relatively high; it is unknown whether plants will take up NAs from environments where the NA concentrations are more similar to those observed in OSPW (1 to 110 mg L^{-1}). In addition, the study revealed that plants can take up NAs from the roots. However, with only investigating the xylem sap there is no evidence on the distribution or eventual fate of these compound in other parts of the plant. Also, Kamaluddin and Zwiazek (2002) studied only poplar seedlings; uptake of NAs may be different for aquatic macrophytes and phytoplankton as well as more mature plants. There are some other concerns with these findings which are previously mentioned in Section 1.3.4, pg 12.

1.6 Plant Biotransformation of Xenobiotics

Biotransformation is a term given to the process whereby compounds which are endogenous or exogenous to an organism (xenobiotics) are transformed by enzymes into metabolites capable of elimination or storage. In mammals, these processes occur mainly in specialized organs (e.g. liver and kidney); however, plants do not contain such organs and biotransformation has been found to occur in all cell types (Durst, 1991). Biotransformation occurs through a multiphase process which transforms the parent compound into more polar products and insoluble bound residues. In Phase I, specialized enzymes convert biologically active toxicants into less active chemicals via hydrolysis and oxidation but occasionally this process can convert the compound to a more toxic metabolite (bioactivation). In Phase II involves the conjugation of a Phase I product with a plant product such as sugars, amino acids, glutathione, and other small molecules. Phase III of biotransformation converts the Phase II product into secondary conjugates or insoluble bound residues. In some plants and chemicals Phase III is the last phase of the biotransformation process; however, in some cases xenobiotics will enter into Phase IV. In Phase IV a Phase II product is conjugated again with another metabolite (e.g. malonic acid) or the glutathione conjugate from Phase II is degraded via peptidases to cysteine conjugates. The product then enters Phase IV where it is then compartmentalized via pump-mediated transport into the plant cell vacuole and/or deposited into the extracellular matrix for incorporation into the cell wall (Sanderman, 1994; Coleman et al., 1997; Korte et al., 2000; Sandermann, 2004).

The enzymes involved in biotransformation have been identified to be located in the microsomal fraction of plant cells, that is the cellular membranes that settle between 10,000 and 100,000 g during centrifugation (Durst, 1991). The microsomal fraction can include organelles such as the smooth and rough endoplasmic reticulum, peroxisomes, tonoplast, and plasmalemma. The biotransformation enzyme classes identified in plants so far include: Cytochrome P450s, glutathione *S*-transferases, carboxylesterases, *O*-glucosyltransferases, *O*-malonyltransferases, *N*-glucosyltransferases, and *N*-

malonyltransferases. Of these enzymes the following enzymes, cytochrome P450s, glutathione *S*-transferases, *O*-malonyltransferases, *N*-glucosyltransferase and *N*-malonyltransferases have been identified previously to be involved in the biotransformation of endogenous and/or exogenous organic acid compounds (Sandermann, 1994) and therefore may be candidate enzymes for NA biotransformation in plants. These enzymes have all been identified to be inducible as in the case of mammals (Durst, 1991) and the genetic modification of plants for phytoremediation can involve the increased production of these enzymes or even insertion of candidate enzymes from other species including mammalian cytochrome P450 enzymes (Arthur et al., 2005; Eapen et al., 2007).

1.7 Constructed Wetlands for Contaminated Water Treatment

Gschöβl and coworkers (1998) investigated the ability of constructed wetlands to polish effluent from municipal wastewater treatment lagoons. In particular the goals of the constructed wetland in treating domestic wastewater was to remove excessive algae growth in the lagoons which can result in increase in water pH which can hamper biocenosis* in the lagoons and effluent receiving waters. Algae are also a suspended solid and therefore can also exert an increase in oxygen demand aquatic systems. The constructed wetlands investigated by Gschöβl and coworkers (1998) used varying combinations of a plastic liner with gravel (d = 5-8 cm) 35 cm deep with a 20 cm free water on top. The macrophytes in these wetlands included bulrush (*Scirpus lacustris*), common reed (*Phragmites australis*) and cattail species (*Typha* spp.). Overall, they confirmed the hypothesis posed by Brix (1994) that macrophytes are able to stabilize surface water and substrate in constructed wetlands, improve conditions for water filtration, and prevent flow systems from clogging, insulate against frost during winter and finally their root systems provide a large surface area for attached microbial growth.

* Biocenosis is a group of interacting organisms that live in a particular habitat and form an ecological community.

In addition the constructed wetlands were capable of buffering the water pH and temperature making the constructed wetlands more favorable for biocenosis.

Gschöβl et al. (1998) concluded that constructed wetlands with larger surface areas and slow flow conditions performed better than those with smaller surface area and faster flow. Soil and indigenous microbial communities were also found to be key in maintaining appropriate nitrogen levels in the wetland for plant growth (Gschöβl et al., 1998). Along with the benefits of macrophytes in wetland systems for treating waste water effluent, the excess of organic matter typical in wetlands supports higher microbial activity than other ecosystems. The latter is also important for breaking down a number of organic contaminants into innocuous byproducts carbon dioxide (CO_2), water, and biomass (CAPP, 2001). As for the application of constructed wetlands for the treatment of OSPW, both Murray-Gulde et al. (2003) and Kanagy et al. (2008) found that constructed wetlands were capable of treating different produced waters from the oil and gas production industries.

1.7.1 Oil sands process water and plant growth

In addition to NAs, OSPW is high in other potentially phytotoxic constituents such as salts (Mikula et al., 1996; Bishay, 1998). Increased conductivity in tailings pond water exerts osmotic stress on the plant which in turn results in water stress in the plant. The influence and effects of salinity in conjunction with NAs; however, was outside the scope of the present study. With regards to the toxicological effects of NAs and salinity, Hayes (2005) found that NA and salinity exert distinct individual effects as well as combined effects on phytoplankton. Cooper (2004) investigated the ability of vegetation communities to develop on reclaimed oil sands wetlands. Consolidated tailings were found to support plant growth as indicated by rapid colonization of isolated CT plots in reference wetlands; however, these wetlands were found to have reduced seedling emergence from seed banks (Cooper, 2004). Baker (2007) found that the application of waste coke (a carbonaceous solid derived from the refining of oil in the cracking process) to stabilize clay-dominated mine tailings in constructed wetlands did not significantly effect macrophyte growth.

1.7.2 Emergent macrophytes

Three native emergent macrophytes were tested for NA uptake and phytotoxicity in the present study: cattail (*Typha latifolia*), common reed (*Phragmites australis*), and bulrush (*Scirpus acutus*). The benefit of using these emergent macrophyte species for phytoremediation is that they are common, prolific, as well as easy to grow and transplant. These species all produce an extensive root system (particularly cattail and common reed) providing greater opportunity for interaction with contaminants as well as surface area for microbial growth and subsequent biofilm production. These species are all capable of covering large areas of shallow water (< 1 m) quickly. Finally these species all have high transpiration rates, which are desired for phytoremediation of contaminated water thereby increasing the rate at which they 'filter' and dewater contaminated water like OSPW.

Cattail (*Typha latifolia*) – is a native, erect aquatic perennial growing up to 3 m in height with creeping white rhizomes. The leaves are flat and 8 – 20 mm in width. Cattail is widespread throughout North America and found in wet or saturated aquatic environments with water depths between 15 – 50 cm. Cattail will tolerate a broad range of climatic conditions and can occupy 58 m^2 within 2 years of seed establishment. Regeneration of cattail primarily occurs through rhizome growth (Johnson et al., 1995; Mitich, 2000).

Common reed (*Phragmites australis* subsp. *americanus*) – has extensively spreading rhizomes with annual cane-like stems that can reach 6 m in height and are 4-10 mm in diameter. Leaves are smooth, alternate, and 20-70 cm long. Along with fresh water ecosystems, common reed can grow in salt-water marshes. The plant clone is extended by perennial rhizomes. Common reed can grow rapidly up to 4 cm d^{-1}. This species is common across North America and into circumpolar regions (Johnson et al., 1995; Mal and Narine, 2004). This species has two closely related sub species the native *americanus* and the non-native *australis*. Unfortunately, the non-native European sub species is more vigorous than the native sub species and can become a noxious weed threatening native flora (Hansen et al., 2007). Nevertheless, common reed is known to grow well in alkaline and brackish conditions; similar to the water conditions observed in tailings ponds (Mal and Narine, 2004). Common reed also has an extensive global

distribution (Hansen et al., 2007). Because of these two traits it is therefore anticipated that this species may do well in OSPW which is alkaline and brackish as well as be able to grow in the range of climates within the region of the Alberta oil sands as well as potential applications to treat OSPW in other countries with oil sands.

Hard-stem bulrush (*Scirpus acutus*) – is a perennial emergent macrophyte with scaly extensive creeping rhizomes. The plant reaches 0.5 to 3 m tall with round soft stems that can be up to 20 mm thick. Bulrush can grow up to 1 m deep in water and is found in a variety of aquatic environments (sloughs, ponds, lakes, marshes, etc.) across North America. This species is tolerant of alkali conditions. Hard-stem bulrush is often one of the earliest rooted macrophytes to invade a flooded area and an important primary producer in aquatic environments (Dabbs, 1971; Johnson et al., 1995).

1.7.3 Hydroponic versus substrate planted plants

The present study used hydroponic systems to test the dissipation and phytotoxicity of NAs in emergent aquatic macrophytes. The purpose of this was to isolate any observed dissipation of NAs to that of the plants. In the presence of substrate there could be excess sorption of NAs in the system to the substrate. Another purpose for using the hydroponic medium was to optimize plant growth and ensure phytotoxicity is a result of the NAs and not due to nutrient deficiencies. Hoagland's nutrient medium was used as it has been developed to provide adequate nutrients and micronutrients to plants (Wetter and Constabel, 1982). Furthermore, it has been used in many studies to assess the fate of environmental chemicals in emergent wetland plants (e.g. Kamaluddin and Zwiazek, 2002; Gallon et al., 2003; Doucette et al., 2005). The effect of NAs observed in plants is not likely to be a result of nutrient deficiencies in the matrix because such deficiencies would be also evident in the untreated control plants.

The present study uses controlled growth conditions for growing plants. However under field conditions there are several factors that could influence the remediation behaviour of the plants. Under deeper water conditions cattail was found to have thicker rhizome diameters and higher concentrations of non-structural carbohydrates compared to cattails planted in shallow conditions (Sharma et al., 2008). This was hypothesized to be a tolerance strategy to maintain aeration of the rhizosphere and better anchorage (Sharma et al., 2008). Common reed was found by Van der Putten et al. (1997) to grow poorly in substrates made up of its own litter; likely a result of phytotoxic compounds

released from decomposing litter. Li et al. (2004) identified that cattail had enhanced biomass production in continuous flooding conditions rather than periodic flooding. Van Bodegom et al. (2008) identified that water depth (flooding) and soil oxygen demand were the two most influential parameters in cattail growth. In the present study these two parameters were controlled by 1) supporting the plants so that only the rhizomes and roots were submerged, 2) oxygenating the hydroponic medium to provide optimal oxygen for the plant roots.

1.8 Microbial Degradation of Naphthenic Acids

In many phytoremediation situations it is not the sole work of plants to perform the remediation. More often than not, particularly in the case of organic contaminants, remediation occurs through a combined plant-microbial system that metabolizes organic compounds and breaks the compounds down into their benign constituents (Arthur et al. 2003). In many cases, the plants play an indirect role in the remediation process through the provision of an extensive surface area on their roots to which bacteria can adhere to in biofilms and metabolize contaminants of concern. As well, plant roots release exudates that can attract and support microbial activity in the rhizosphere area (Dakora and Phillips, 2002). Plant health in contaminated environments is supported with a healthy rhizosphere microbial community which can metabolize toxic compounds prior to reaching the plant, provide better assimilation of nutrients, as well as protection from pathogenic bacteria. It is not known what role microorganisms play in the rhizosphere of wetland plants exposed to organic contaminants in particular those exposed to oil sands NAs and OSPW.

Foght et al. (1985) reported aerobic microbial content at 0.5 m depth to be around 1.3×10^6 colony forming units mL^{-1}; and anaerobes and sulfate-reducers at 1.5×10^3 and 1.2×10^5 most probable number mL^{-1} respectively in a Syncrude tailings pond. Mineralization studies indicated that the microbial populations are capable of mineralizing simple organic compounds in particular those bacteria measured in shallow samples (0.5 m) had much higher rate of hydrocarbon-degrading capability as measured

through labeled phenanthrene and hexadecane. They attributed this degradation to the predominance of *Alcaligenes* spp. which was identified to be present in OSPW (Foght et al. 1985).

Herman et al. (1994) determined the metabolic capabilities of enrichment cultures using model compounds that represented the cycloalkane ring and the aliphatic side chain of NAs. The NAs degrading enrichment culture was found to be specialized for the mineralization of compounds containing carboxylic acid groups but showed no activity against hexadecane. This indicates that specialized bacterial communities are required to degrade NAs for remediation. Biodegradation of the model NA compounds showed that NA-degrading bacteria did this by oxidizing the carboxylated aliphatic side chain which eventually led to the oxidation of the cycloalkane ring. The authors also determined that the plant nutrients nitrogen (N) and phosphorus (P) were also able to stimulate microbial activity (Herman et al., 1994). This means that plants could be in competition for nutrients with bacteria.

Lai et al. (1996) also found that OSPW contained microorganisms well adapted to mineralizing NAs. They found that decreasing dissolved oxygen (DO) and temperature followed the rate of degradation of these compounds and addition of phosphate increased the rate of degradation and the rate of oxygen consumption. Therefore, because aquatic macrophytes are known to increase oxygenation at the root surface (Matsui and Tsuchiya, 2006) plants may provide support for microbial degradation of NAs simply through the process of supplying oxygen for microbial respiration. In their studies, Lai et al. (1996) also did not find reduction in toxicity of OSPW with incubations with bacteria isolated from OSPW for up to 8 weeks.

Clemente et al. (2004) investigated further the aerobic biodegradation of two commercial NA preparations and found that commercial preparations can be biodegraded extensively under laboratory conditions. However, the degradation of NAs in the environment is predicted to be slower as a result of the limitation of oxygen and nutrients (N and P). The oil sands NA mixture is also more complex than that of a commercial preparation, which could also slow down NA biodegradation (Clemente et al., 2004). Scott et al. (2005) confirmed that the OSPW NAs were less biodegradable than the commercial NA mixtures (20% vs. 48%) because commercial NA mixtures were made

up of lower molecular weight NAs. Moreover, in their review of the topic, Clement and Fedorak (2005) identified that minimal work has been done on the potential of bacteria to degrade oil sands specific NAs. Most work has been conducted on single model NA compounds or with commercial NA mixtures.

Videla (2007) investigated the stable isotopes of ^{13}C and ^{15}N in static and semi-continuous laboratory microcosms for utilization of commercial NAs by oil sands derived microbial cultures. Degradation of NAs was found to be a result of a variety of factors including the initial concentration of NAs, source of the NA mixture, exposure duration, as well as nutrients and dissolved oxygen levels. Videla (2007) found that microbial biomass was similar or ^{13}C enriched (0.3 to 2.9 ‰) relative to the dissolved organic carbon (DOC) source, depending on the length of incubation. In contrast, it was found that utilization of the oil sands NA extract resulted in greater ^{13}C enrichment of microbial biomass (3.7 to 8.5 ‰) relative to the DOC source. Overall, the $\delta^{13}C$ of the DOC comprised of NAs showed minimal change (-0.5 to +0.2 ‰) during the incubation period whereas the $\delta^{13}C$ of the dissolved inorganic carbon (DIC) was more variable (-5 to +5.5 ‰). Finally, it was found that in some semi-continuous tests, the final $\delta^{15}N$ biomass values were ^{15}N enriched (3.8 to 8.4 ‰) relative to the initial biomass (Videla, 2007).

Hadwin et al. (2005) identified using denaturing gradient gel electrophoresis (DGGE) data that bacterial communities in wetland sediments clearly separated according to NA concentrations. These DGGE profiles also showed that these bacterial communities in NA exposed wetlands were homogeneous and that these communities found within wetland sediments were capable of degrading NAs. All wetland sediments tested were found to degrade monocyclic model NA compounds but only those microbial communities from sediments which had been previously exposed to OSPW were capable of degrading bicyclic model NA surrogate. Hadwin et al. (2005) also reported that not only did the microbial community change between those sediments exposed to NAs from OSPW but that a sediment sample collected from a wetland with the highest NA and oil concentrations as well as lacking plant life had a microbial community even more unique than the other samples. This indicates that in the absence of plants, sediment samples do not establish the same microbial community. These authors also reported that even wetlands with low impact contained a microbial community with a shift to one capable of

metabolizing NAs. However, the time required to shift the microbial community in a wetland to one that metabolizes NAs is unknown. Therefore, it may prove advantageous to 'seed' wetlands intended for remediation with sediment from those areas where the microbial communities are known to have a high remediation potential.

With regards to phytoremediation, the microbial community of most interest is that which lives within the rhizosphere (~1 cm zone around the roots) on the root surface (rhizoplane bacteria) and within plant roots (endophytic bacteria). Biryukova et al. (2007) investigated rhizosphere microbial degradation from rhizosphere communities isolated from various native boreal terrestrial plants: labrador tea (*Ledum groenlandicum*), awned haircap moss (*Polytrichium piliferum*), paper birch (*Betula papyrifera*), bluejoint (*Calamagrostis canadensis*), and foxberry (*Vaccinium vitis-idaea*). Here the authors found that these isolated rhizosphere bacterial communities were capable of degrading more than 90% a the original starting concentration 135 mg L^{-1} commercial NA mixture (Merichem, Houston, TX, USA) over a period of 10 days to 90% of the starting concentration paired with a 100-fold increase in the colony forming units (CFU) during that time. Similar to many biodegradation studies non-cyclic NAs (Z = 0) were more susceptible to biodegradation than the cyclic acids.

1.9 Use of Freshwater Macrophytes for Phytotoxicity Testing

Historically freshwater plants were considered less sensitive than animal species which is why toxicity testing favoured the use of invertebrates and fish. In actuality, sensitivity is chemical- and organism- specific and therefore phytotoxicity data should be a necessary part of the hazard assessment process (Lewis, 1995). In addition, when macrophytes are actually tested, the floating macrophyte duckweed (*Lemna* spp.) is frequently used as a representative of all other aquatic vascular plants. Although this small species is ideal for experimentation, duckweed are floating plants and are unattached to the substrate which could overlook an important avenue of contaminant exposure (Lewis, 1995). As future remediation strategies for OSPW includes a wetlandscape approach (van den Heuvel, 1999), toxicity investigations of the primary

producers in such scenarios are necessary. Aquatic plants not only oxygenate sediments but also provide habitat for many other aquatic species integral to functioning wetlands (invertebrates, fish, etc.) (Johnson et al., 1995)

Hanson et al. (2003) investigated the use of 10 different aquatic plant endpoints in the submergent macrophyte milfoil (*Myriophyllum* spp.) and determined which of these endpoints showed the greatest sensitivity and least variability to monochloroacetic acid (MCA), dichloroacetic acid (DCA), chlorodicluoroacetic acid (CDFA). Pigment concentrations (chlorophyll *a* and *b*) were found to be variable between species, whereas plant length and wet mass measurements were found to have the greatest statistical sensitivity, ecological relevance, and sensitivity to toxicants (Hanson et al., 2003). However, morphological endpoints do not react quickly to the introduction of contaminants and are difficult to measure throughout the course of the experiment. As well, in the case of cattail which tends to send out more shoots rather than grow in length, shoot length is difficult to determine. Trapp et al. (2000) was able to address both of these problems (reaction time and physiology constraints for shoot length) by using transpiration as a toxicity endpoint. Hanson et al. (2003) also determined that the variability of field studies in mesocosms with the macrophyte *Myriophyllum* spp. was not significantly different ($P < 0.05$) from that of laboratory studies. This is important to consider in the application of the results obtained in the presant laboratory based research to the natural environment. Finally, the authors established the benchmark of EC_{25} for aquatic macrophytes to cause an ecologically significant effect (Hanson et al., 2003). This benchmark can be applied to the present study to determine if plants can persist in a NA phytoremediation system, outside of a controlled growth chamber environment.

1.10 Research Objectives

The overall objective of this research was to determine fate and effects of oil sands NAs in emergent macrophytes. *It was hypothesized that emergent macrophytes would enhance the dissipation of NAs from hydroponic medium through uptake, biotransformation of NAs, and eventual incorporation into plant tissue as well as through supporting rhizosphere microorganisms which would also enhance NA dissipation.* To meet this objective the following sub objectives were identified:

1) Develop a hydroponic testing system for the evaluation of NA dissipation and phytotoxicity in emergent macrophytes.

2) Assess the phytotoxicity of NAs in emergent macrophytes.

3) Determine the dissipation of NAs and toxicity reduction in systems planted with emergent macrophytes.

4) Determine the role rhizosphere bacteria play in emergent macrophyte NA phytotoxicity.

5) Investigate methods to detect NAs in plant tissue to determine the fate of NAs in plant tissue.

6) Evaluate the toxicity and NA dissipation in oil sands fine tailings treatments.

Table 1.2: Research objectives by chapter.

Chapter	Objectives	Description of Chapter
1	Introduction and Background	▪ Information on NA chemistry in oil sands process water and NA environmental fate. ▪ Current knowledge on NA toxicity. ▪ Analytical methodologies for NA analysis with specific focus on analysis in hydroponic medium and plant tissue. ▪ A review on plant uptake of chemicals and biotransformation and the use of constructed wetlands for phytoremediation of contaminants.
2	To develop a hydroponic testing system for the evaluation of NA dissipation and phytotoxicity in emergent macrophytes. To assess the phytotoxicity of NAs in emergent macrophytes.	▪ Experiments conducted to optimize hydroponic experiments and phytotoxicity endpoints, sample analysis. ▪ Hydroponic experiments conducted to compare the phytotoxicity and dissipation of a commercial NA extract to a NA extract from oil sands process water. Published: *Journal of Environmental Science and Health, Part A (2008), 43:36-42.*
3	To assess the phytotoxicity of NAs in emergent macrophytes. To determine the dissipation of NAs and toxicity reduction in systems planted with emergent macrophytes.	▪ Hydroponic experiments conducted to determine the phytotoxicity and dissipation of ionized and non-ionized NAs. ▪ Acute *Daphnia magna* 48 h LC_{50} toxicity tests were performed on NA hydroponic medium pre- and post-phytoremediation treatment and compared to unplanted treatments to assess toxicity reduction.
4	To determine the role rhizosphere bacteria play in emergent macrophyte NA phytotoxicity.	▪ Bulk water, root surface, and root samples collected from plants exposed to NAs in hydroponic medium. DNA extracted for analysis using molecular techniques for the assessment of changes to the microbial community structure as a result of NA exposure.
5	To investigate methods to detect NAs in plant tissue to determine the fate of NAs in plant tissue.	▪ Experiments conducted using pressurized liquid extraction of plant tissue with analysis of liquid extracts by electrospray ionization – mass spectrometry for the analysis of NAs in plant tissue. ▪ Experiments conducted at the Canadian Lightsource Synchrotron for the analysis of NAs and biochemical changes across root cross sections, using FTIR microspectroscopy.
6	To evaluate the toxicity and NA dissipation in oil sands fine tailings treatments	▪ Hydroponic experiments conducted with oil sands fine tailings, fine tailings amendments, and simulated runoff water from dried fine tailings, to determine phytotoxicity and performance of emergent macrophytes under more relevant remediation effluent scenario.
7	General discussion, conclusions and recommendations	▪ A summary of the results obtained in each chapter with a summary of key findings and conclusions. ▪ A comprehensive list of suggested future research directions.
8	References	

2.0 Phytotoxicity of Oil Sands Naphthenic Acids and Dissipation from Systems Planted with Emergent Aquatic Macrophytes

2.1 Preface

This research has been published (reference cited below); a modified version is included in this thesis with the authors' permission.

Armstrong, S.A., Headley, J.V., Peru, K.M., Germida, J.J. 2008. Phytotoxicity of oil sands naphthenic acids and dissipation from systems planted with emergent aquatic macrophytes. *Journal of Environmental Science and Health Part A*, 43:36-42.

2.2 Introduction

As a group, naphthenic acids (NAs) are classified as organic acid compounds composed predominately of alkyl-substituted cycloaliphatic carboxylic acids and to a lesser extent acyclic aliphatic acids. The general chemical formula for NAs is $C_nH_{2n+z}O_2$ where n is equal to the number of carbon atoms, and Z is equal to zero or a negative even number and represents the number of hydrogen atoms lost as a result of the structures becoming more compact (Fan, 1991). Naphthenic acids are considered to be surfactants because they have both a hydrophilic component (carboxyl group) and a hydrophobic component (non-polar aliphatic group) (Acevedo et al., 1999).

Naphthenic acids are released from bitumen during the mining of oil sands and the processing of bitumen for oil production. Thus, NAs are found in the large volumes of oil sands process water (OSPW) produced at oil sands operations in Fort McMurray, Alberta, Canada. Naphthenic acids are of concern in OSPW because they can subsequently migrate into aquatic environments (Bendell-Young et al., 2000; Crowe et

al., 2001). In addition to their water solubility and sorption to soils, NAs are also of toxicological concern in the environment because they have acute aquatic toxicity to a variety of aquatic organisms including fish (Headley and McMartin et al., 2004; Clemente and Fedorak, 2005).

Investigations on the factors driving the fate and toxicity of NAs in plants have received little attention to date (Crowe et al., 2001; Kamaluddin and Zwiazek, 2002; Apostol et al., 2004). Nix and Martin's (1992) research on the effect of OSPW on the algae *Pseudokirchneriella subcapitata* (formerly know as *Selenastrum capricornutum*) have shown that tailings pond water can be stimulatory. Stimulation of plant metabolism by some sodium naphthenates was also observed by Wort and colleagues (1973) in experiments with foliar application on *Phaseolus vulgaris*. Work with the aquatic macrophyte cattail (*Typha latifolia*) indicated that wetland plants are adaptive to OSPW (Crowe et al., 2001). In contrast, laboratory studies with a commercially available NA mixture using hydroponically grown poplar saplings found that NAs were acutely toxic (Kamaluddin and Zwiazek, 2004). These apparent differences in results reported in the literature highlights the need for isolation and identification of the individual NA compounds present in a given NA mixture (Crowe et al., 2001). Information on the mode of phytotoxicity of specific NA compounds is not currently available.

In this study, a variety of common native emergent aquatic macrophytes [cattail (*Typha latifolia*); common reed grass (*Phragmites australis*); and hard stem bulrush (*Scirpus acutus*)] were exposed to NAs hydroponically in controlled laboratory conditions. The present study investigates the differences in phytotoxicity and the dissipation of a commercial NA mixture compared to a NA mixture extracted from OSPW. Phytotoxicity was measured by monitoring the uptake of hydroponic medium from the testing vessel as traditional plant health parameters (shoot and root length, plant weight gain) were not appropriate. For example, shoot and root length were not used because cattails and bulrush only grow to a specific length and then send out new growth as side shoots. Likewise, plant weight can only be monitored at Day 0 and Day 30 as it is too stressful on the plants to monitor fresh weight gain throughout the experiment on multiple days. Water relations in plants are; however, a key parameter reflecting plant health. This property is therefore used here to monitor the phytotoxicity of NAs to plants

(Trapp et al., 2000; Doucette et al., 2005).

2.3 Materials and Methods

2.3.1 Chemicals and materials

Fluka commercial naphthenic acid mixture (Fluka NAs) was obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Unless otherwise noted, all other chemicals and materials used in the growth chamber experiment, sample clean up, and analysis were obtained from Fisher Scientific (Edmonton, AB).

2.3.2 Oil sands naphthenic acid extract preparation

An oil sands NA extract was prepared from approximately 500 L of OSPW collected in July 2005 from an oil sands extraction operation (Fort McMurray, AB, Canada). The NAs were extracted from OSPW using an adapted liquid-liquid extraction method described by Rogers et al. (2002a) and Janfada et al. (2006). An approximate volume of 3 L final extract was produced with an NA concentration of 6,800 mg L^{-1}. This final NA extract was prepared in 0.1 N KOH with the pH adjusted to 8.0 using 18.76M H_2SO_4. The concentration of 6,800 mg L^{-1} was determined by comparison to an aliquot of the oil sands NA extract produced by Rogers et al. (2002a). The aliquot from Rogers et al. (2002a) was serially diluted and then a five-point linear regression curve was created for quantification of unknown NA extracts. The quantification of the oil sands extract was further verified by comparing the area counts of the oil sands NA extract with the area counts produced by the same concentration of commercially available Fluka NA extract. The area counts of the two samples correlated well and confirmed the concentrations determined using the calibration curve created with the NA extract produced by Rogers et al. (2002a).

2.3.3 Hydroponic experiments

Plants were obtained as root cuttings from a native wetland plant nursery (Bearberry Creek Water Gardens, Sundre, AB, Canada) in June 2005 for the commercial NA experiments and October 2005 for the oil sands NA experiments. Root cuttings were mass cultured for at least three weeks in plastic containers with ¼ strength modified Hoagland's nutrient medium (235 mg L^{-1} $Ca(NO_3)_2.4H_2O$; 130 mg L^{-1} $MgSO_4.7H_2O$; 165 mg L^{-1} KNO_3; 30 mg L^{-1} $NH_4H_2PO_4$; 17.5 mg L^{-1} Sequestrene 330 Fe (Fe-DTPA); with 0.1 mL of the following micronutrient solution per liter of medium [7 g L^{-1} H_3BO_3; 8.5 g L^{-1} $MnSO_4.H_2O$; 0.25 g L^{-1} $CuSO_4.5H_2O$; 0.55 g L^{-1} $ZnSO_4.7H_2O$; 0.25 g L^{-1} $(NH_4)_6Mo_7O_{24}.4H_2O$; and 1.25 mL of H_2SO_4 (conc.)]) in an environmental chamber. The environmental chamber was maintained at a 16:8 hour, 22°C:18°C day/night cycle, an average light intensity of 12,000 lux, and an average relative humidity of 30%:35% for day and night respectively. Once the plants were acclimatized to the chamber conditions, plants that were ~62 cm in length, 7 cm long rhizome, and ~25.7 g fresh weight (cattail); ~48 cm in length and ~5.6 g fresh weight (common reed); and ~66 cm in length and ~15.0 g fresh weight (bulrush) were set up for two weeks prior to the start of the experiment. Plants were grown in a hydroponic system similar to the apparatus described by Doucette et al. (2005) except 2.5 L amber borosilicate glass jars were used and the system was aerated with the use of aquarium air pumps (Figure 2.1). Planted treatments contained plants in the hydroponic testing system; where as unplanted treatments were not planted and were composed of hydroponic medium in the hydroponic testing system only. Each planted treatment contained three plant replicates (one replicated is equal to one plant in an individual testing system) and there were three different NA treatments (0 mg L^{-1} NAs; 30 mg L^{-1} NAs; and 60 mg L^{-1} NAs) applied on each species investigated. The doses employed were based on the results of preliminary experiments with cattail and Fluka NAs (data not shown). A dose of 60 mg L^{-1} caused acute toxicity in the preliminary trials where as 30 mg L^{-1} was the lowest observed adverse effects level (LOAEL). Two treatments were dosed with 60 mg L^{-1} and served as unplanted controls (for each unplanted treatment, n = 3). One of the unplanted controls was sterilized with 525 mg L^{-1} sodium azide to monitor abiotic loses of NAs to the hydroponic system. The other unplanted control was used to account for biotic loses due to microbiological

communities that may form in the hydroponic media over the course of the experiment. A closed-cell plastic foam which fitted snug to the opening of the test vessel was used to support the plants and to keep the evaporation of hydroponic medium to a minimum. Transpired hydroponic medium was

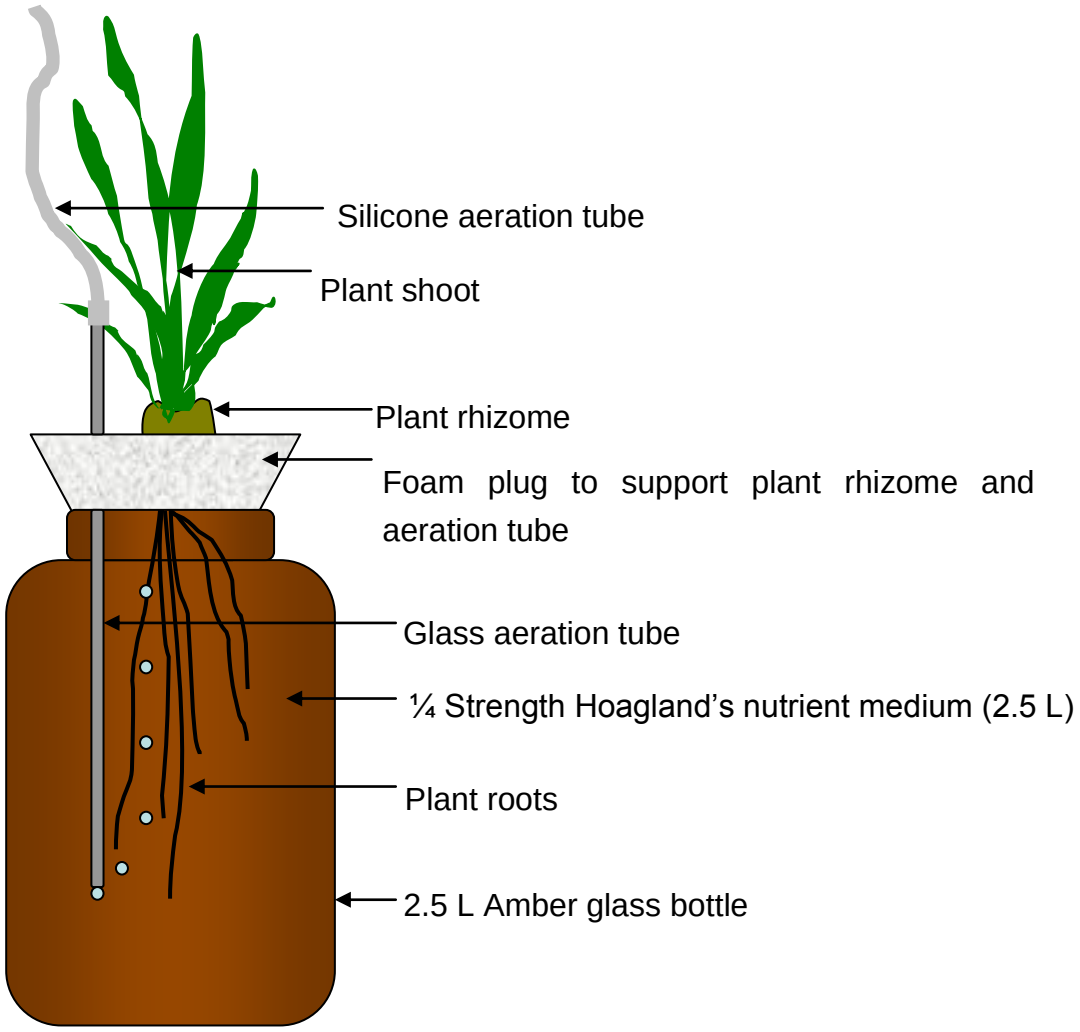

Figure 2.1: Schematic diagram of the hydroponic testing system.

monitored by measuring water uptake (Trapp et al., 2000) and topped up every 5 days. The pH was maintained at 7.8 using 1.0 N HCl or 1.0 N KOH. While being stirred with a magnetic stir bar, a 2 mL hydroponic medium sample was taken using a pasteur pipette on Day 0 immediately after spiking with NAs and then again on Days 5, 10, 20 and 30. Plant wet weights were recorded on Days 0 and 30 to monitor plant growth over the

course of the experiment.

2.3.4 Analysis

Hydroponic medium samples were analyzed for NAs using a Quattro Ultima (Waters Corp. Milford, MA, USA) triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface operating in the negative ion mode. Instrument operating parameters are reported elsewhere (McMartin et al., 2004; Appendix A).

Preliminary studies found that hydroponic medium samples could not be directly injected for analysis due to background ion interference (Appendix B). Samples were therefore cleaned up using ENV+ solid phase extraction (SPE) cartridges (Biotage, Chartlottesville, VA, USA) (Headley et al., 2002). A 1.5 mL volume of sample was diluted with double deionized water and acidified using 0.5 mL of formic acid. Samples were run through the SPE column, rinsed with 5 mL water, and then eluted using 6 mL acetonitrile. The acetonitrile eluant was then evaporated to dryness under a N_2 stream. The final sample was then reconstituted with 1.0 mL of 50:50 acetonitrile:water and 0.1% NH_4OH, sonicated for 30 seconds, and then transferred to a 2 mL amber LC vial (Waters, Milford, MA, USA).

2.3.5 Selective uptake

In addition to measurements of total NA uptake, an investigation was conducted to determine if plants selectively take up individual NA compounds from the NA mixture. The relative abundance of each NA within the mixture was determined by arranging the mass spectrum data according to carbon number and Z family. This method of analysis was modeled after Holowenko and coworkers (2001).

2.3.6 Data analysis

Data were tested for normality and homogeneity of the variances using Anderson-Darling and Levene's tests respectively. Data that did not meet the assumptions of normality and homogeneity of variances was log-transformed. Data were then tested for

differences between treatment means using an analysis of variance (ANOVA). For each species and NA mixture, sampling day was used as a factor with NA concentration as the dependent variable. Then for each species and NA mixture on Day 30, treatment was used as a factor and NA concentration was used as the dependent variable. To determine the effect of NAs on transpiration within each species for each five day interval, the factor was NA treatment and the dependent variable was water uptake. Specific differences between treatment means were determined using a Tukey test for post-hoc analysis. All statistical analyses were carried out using Minitab Release 13 (Minitab Inc. State College, PA, 2000). All graphs were created using SigmaPlot 8.1 software (SPSS Inc., Chicago, IL, 2002).

2.4 Results and Discussion

The concentration of both types of NAs mixtures in the hydroponic systems (aqueous phase), appeared to peak from Day 0 to Day 5 in all treatments (Figure 2.2). This increase in concentration with time at the start of exposure is likely the result of a steady state between the NAs in solution and the surfaces of the testing units and has been observed with other organic contaminants (Brusseau et al., 1990; Headley et al., 1998). The NA concentration was ~15-20 % of the nominal spike by Day 5 in all commercial NA treatments (Figures 2.2 a, b, c). This phenomenon has been observed in previous work with the Fluka commercial NA mixture and is likely a result of solubility effects or sorption to either the surface of the borosilicate glass testing units (McMartin et al., 2004), or sorption to carbonate compounds in the media.

The total concentration of oil sands NAs in planted treatments does not appear to dissipate significantly over a 30 day exposure period $P < 0.05$ (Figures 2.2 d, e, f). There appears to be loss of total NAs, however, over the 30 day exposure period with the commercial NA mixture. Although this loss was greater in the planted treatments; there was only a significant difference on Day 30 between the unplanted treatments with 60 mg L^{-1} and the planted treatment containing common reed (Figure 2.2 b). Surprisingly this trend of NA loss over time was not observed in the treatments with 30 mg L^{-1} NAs

(Figures 2.2 a, b, c). The loss of Fluka commercial NAs over time in treatments planted with common reed appeared to be selective to certain NAs (Figure 2.3). In particular the NAs in the Z = -4 series (two-ring NAs) with a carbon number ranging from 12 to 15 displayed the greatest loss of abundance of 36.7 % ± 8.0 % SE (n = 3) from Day 5 to Day 30 (Figure 2.3c and 2.3d). There was a parallel increase in abundance of carbon number 16 to 18 (55.5 % ± 9.6 % SE [n = 3]) of non-cyclic (straight chained or branched) NAs (*Z* = 0) from Day 5 to Day 30 (Figure 2.3c and 2.3d). The decrease in abundance of two-ring (*Z* = -4) series is even more evident when looking specifically at the relative abundance of Z = -4 series in the planted versus unplanted treatment between Day 5 and 30 (Figure 2.4b and 2.4d).

Careful attention must thus be given to appropriate data reduction and processing. For example, the dissipation of selective ions in the Z = -4 series is masked when observing the total NAs (Figure 2.2 vs. Figure 2.4). Likewise, Day 5 was used to compare selective ion dissipation to Day 30 rather than Day 0 because Day 5 was the point of maximum NAs concentration (a result of the equilibrium time required to achieve a maximum concentration of NAs after initial spiking into the testing vessel).

For full verification of plant uptake of NAs, it is desirable to measure the levels of NAs directly in plant tissue. While this analytical method development is a topic of future research, the noted difference in NA concentrations observed in the present study can likely be attributed to the presence of plants as all other parameters within the testing vessel were kept relatively constant, medium, pH, temperature, etc. Furthermore, the differences were compared to unplanted controls. Thus the change observed in the NA medium concentration appears to be primarily due to the presence of plants, and their associated microorganisms. At this point it is not possible to identify whether this dissipation is due to adsorption to the root or absorption into the root, or if the root associated microorganisms are responsible for metabolizing the NAs.

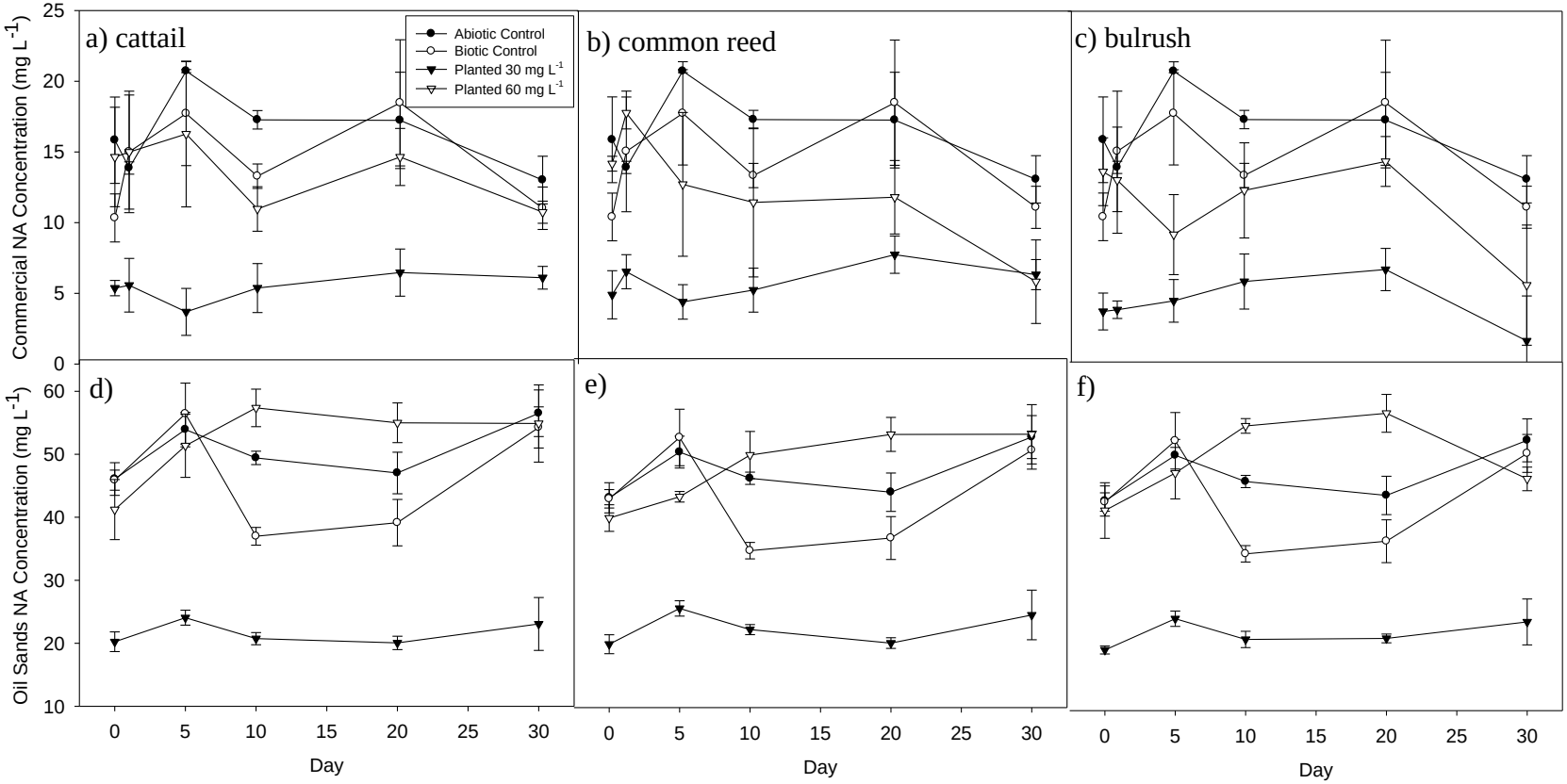

Figure 2.2: Naphthenic acid (NA) concentration (n = 3, ± SE), in hydroponic medium over 30 days for Fluka commercial NAs in a) cattail; b) common reed; c) bulrush and for oil sands NAs in d) cattail; e) common reed; f) bulrush.

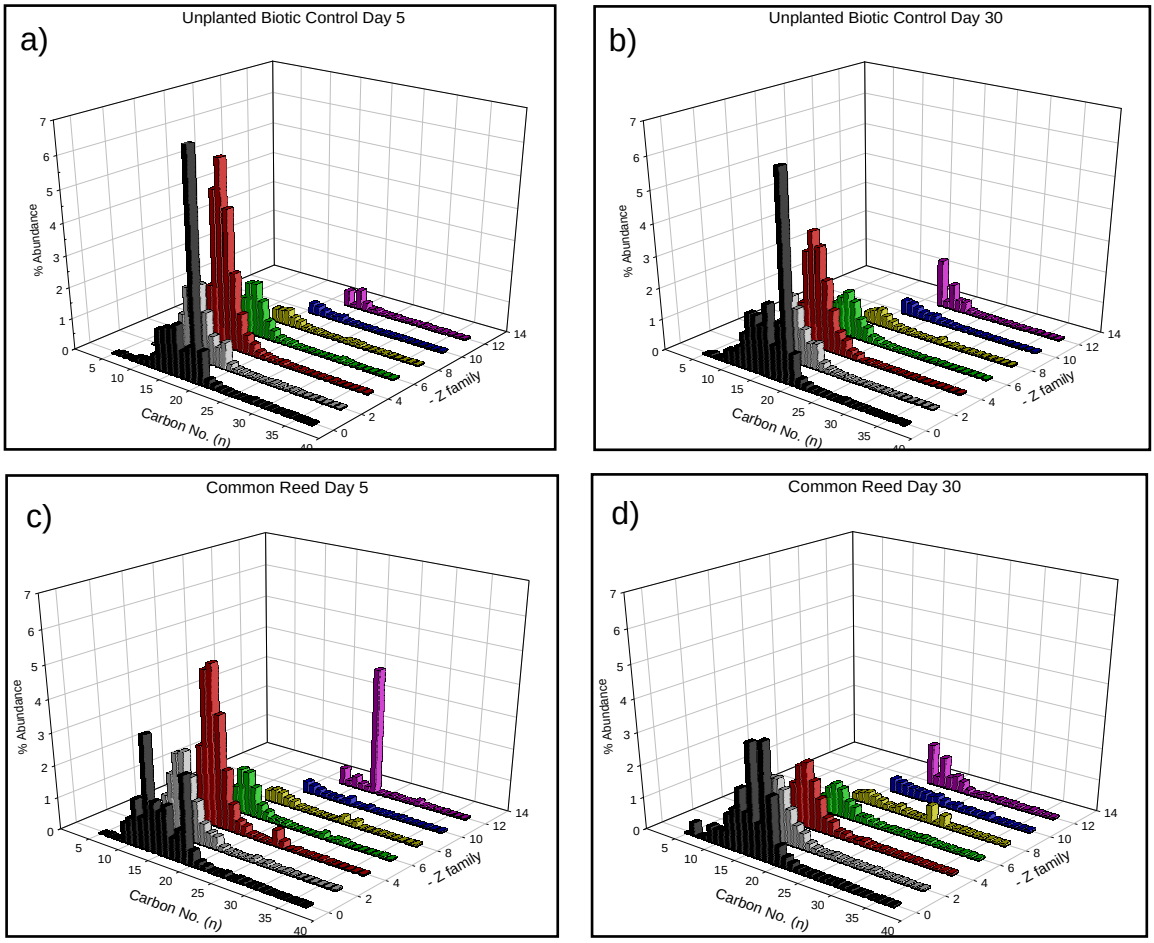

Figure 2.3: Percent abundance of Fluka commercial naphthenic acids (NAs) versus carbon number and –Z family on a) Day 5 and b) Day 30 in unplanted treatments with 60 mg L^{-1} NAs and c) Day 5 and d) Day 30 for treatments with 60 mg L^{-1} NAs planted with common reed. Values are reported as an average of 3 replicates (n = 3). Error bars are not reported here to permit easier visual inspection of the data.

The NA dissipation from the planted hydroponic system is closely mimicked by the toxicity data obtained from wetland plants. Water uptake, used in the present study as a simple technique to monitor the rate of transpiration (Trapp et al., 2000), was found to be the most sensitive parameter in plants exposed to NA. This supports the finding of Kamaluddin and Zwiazek (2002) that NAs are likely to interfere with water relations within the plant. In the present study cattail was found to be the most sensitive species with significantly altered water uptake after 10 days of exposure ($P < 0.05$) (Figure 2.5).

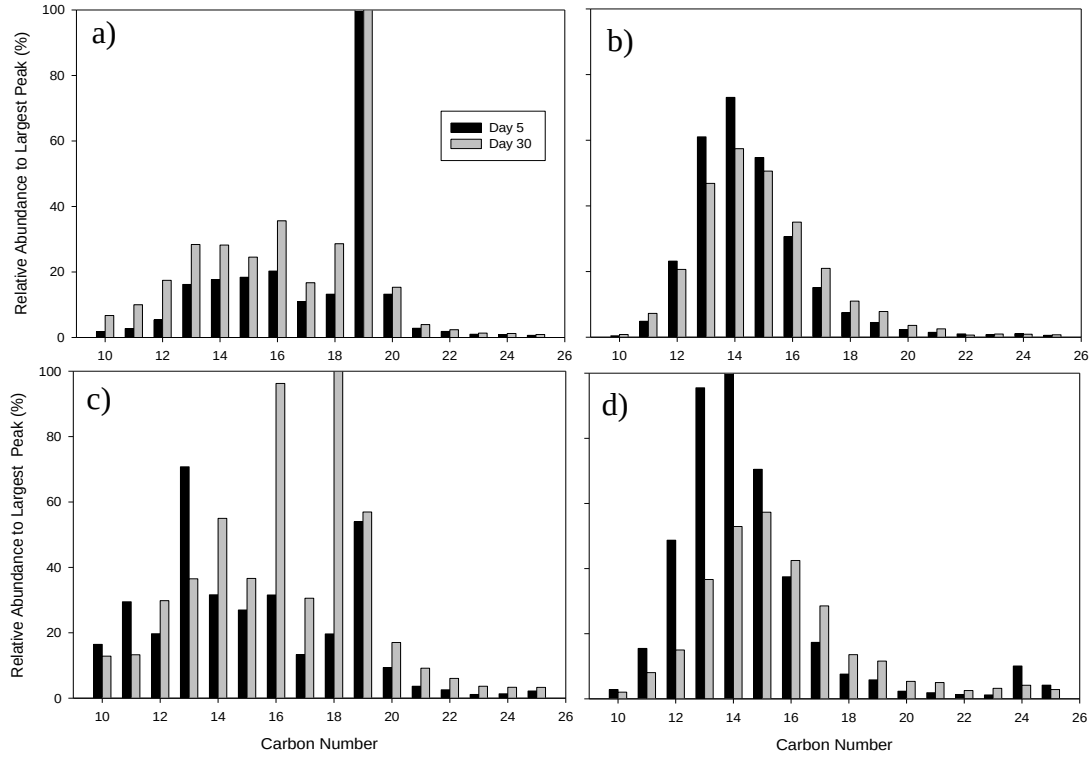

Figure 2.4: Carbon number versus the mean (n = 3) percent relative abundance of the largest mass spectra peak of the sample for unplanted biotic controls, treated with 60 mg L^{-1} of Fluka naphthenic acids (NAs), for the a) Z = 0 and b) Z = -4 family, and for the treatments planted with common reed, treated with 60 mg L^{-1} Fluka NAs, for the c) Z = 0 and d) Z = -4 family.

Common reed was found to be the most tolerant plant species tested, especially to low NA doses (30 mg L^{-1}) (Figure 2.5). This is likely why common reed had the highest dissipation of NAs of the planted treatments dosed with 60 mg L^{-1} NAs (Figure 2.2). There was greater variability in the water uptake in oil sands NA exposed plants which could be due to individual differences in the plants as well as the wide range of compounds contained in the oil sands NA mixture (Figure 2.5 and 2.6).

Figure 2.6 shows a comparison of the mass chromatogram of naphthenic acids in the commercial NA mixture and the mass chromatogram of the oil sands NAs both used in the present study. It is evident from Figure 2.6 that commercial NAs have a lower range of components (157-297 *m/z*) compared to the oil sands NAs (195-325 *m/z*) from

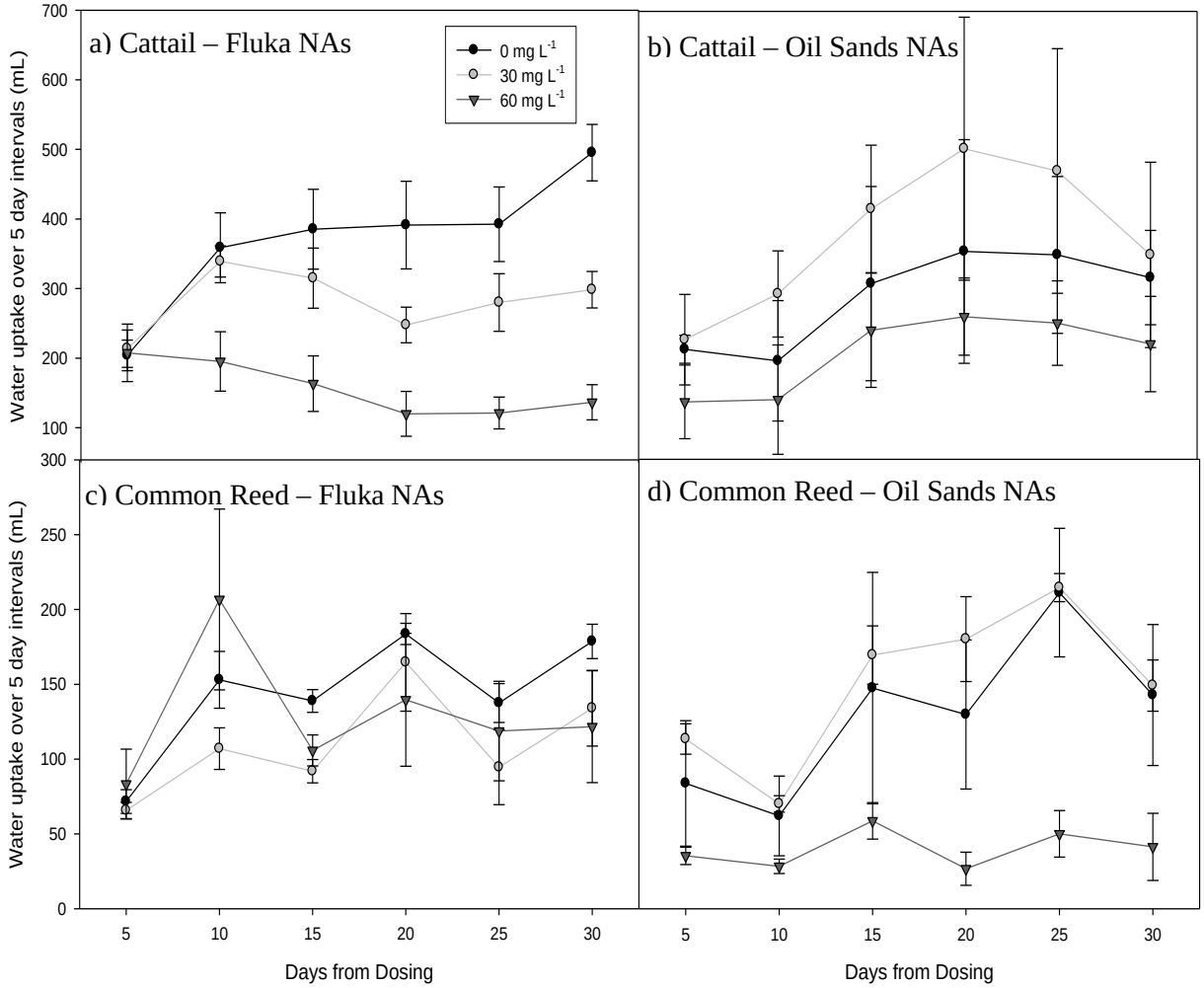

Figure 2.5: Mean water uptake over 5-day intervals (n = 3, ± SE) in hydroponic treatments planted with cattail exposed to a) Fluka commercial naphthenic acids (NAs) and b) oil sands NAs, and in treatments planted with common reed exposed to c) Fluka commercial NAs and d) oil sands NAs.

the Athabasca Basin, Canada. The lower molecular weight NAs appear to be more phytotoxic than the higher molecular weight NAs. However, further investigation into the phytotoxicity of different NA fractions is warranted in view of the complexity of NA mixtures. In addition, because there was evidence of dissipation and phytotoxicity when plants were exposed to lower molecular weight NAs, it can be inferred that lower molecular weight NAs are able to dissipate from hydroponic medium in the presence of

plants either through adsorption or absorption into plant roots. Development of techniques to analyze NAs within plant tissue will help differentiate whether this dissipation is through root adsorption or root uptake. As illustrated by the comparison of water uptake data (Figure 2.5) with the NA uptake in plants for commercial NAs (Figure 2.2), it is evident that if the plant is more tolerant to NAs there is greater NA uptake. This tolerance is exhibited as reduced differences between exposed and unexposed plants in water uptake, as seen in common reed, Figure 2.5. The differences observed between the oil sands NA mixture and the commercial Fluka NA mixture here support the findings observed by Scott et al. (2005).

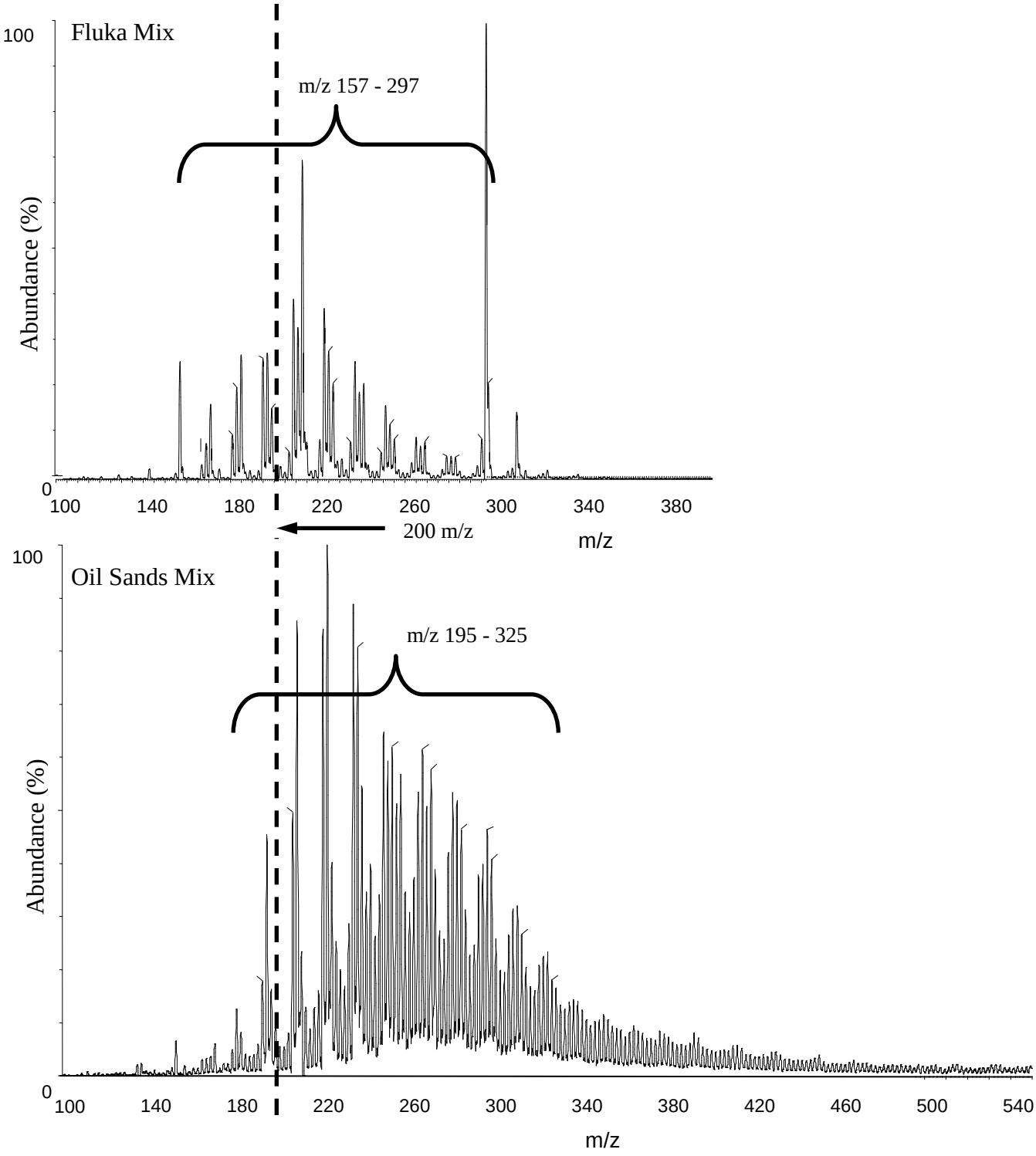

Figure 2.6: Comparison of the mass chromatograms of the loop injection of Fluka commercial naphthenic acid (NA) mixture and of the oil sands NA mixture.

2.5 Conclusions

In summary, the oil sands NA mixture was less toxic to wetland plants compared to the commercially available NA mixture, and were not sequestered by wetland plants like their commercial NA counterparts. The small loss of commercial NAs from spiked hydroponic system was selective and dependent on the specific NA compound. Wetland plants (in hydroponic systems) alone may not therefore mitigate NAs from oil sands tailings pond water. Additionally, caution should be taken when making predictions on the environmental fate of oil sands NAs when using commercial NAs as surrogates.

3.0 Differences in Phytotoxicity and Dissipation between Ionized and Non-ionized Oil Sands Naphthenic Acids in Wetland Plants

3.1 Introduction

Naphthenic acids (NAs) are a group of organic acid compounds that are found naturally in oil sands deposits (Figure 1.1) (Fan, 1991). During the caustic hot-water extraction of oil from the bitumen in oil sands deposits, NAs become concentrated in the resulting oil sands process water (OSPW). Naphthenic acids are found at concentrations ranging from 40 – 120 mg L^{-1} in the OSPW of mining operations in the Athabasca oil sands deposits near Fort McMurray, Alberta, Canada (Holowenko et al., 2000). Naphthenic acids are a concern in OSPW because they are acutely toxic to fish and aquatic invertebrates with an LC_{50} ranging between 4 and 78 mg L^{-1} depending on the species, water hardness, water temperature, length of exposure, and dissolved oxygen concentration (Dokholyan and Magomedov 1983; Verbeek et al., 1994). Additionally, NAs are water soluble at the alkaline pH conditions of OSPW and have the potential to migrate into surrounding aquatic environments (Bendell-Young et al., 2000). The exact chemical composition of compounds in NA mixtures depends on the source of the NAs. For NAs from the Athabasca oil sands, the dissociation constant (pK_a) ranges between 5 and 6 (Headley et al., 2004). Most oil sands tailings pond waters are alkaline and thus NAs will be predominantly in their ionized, water soluble form as naphthenate salts.

The extraction of oil from bitumen is heavily reliant on water, requiring approximately 12 m^3 of water for every 1 m^3 barrel of oil produced and after recycling water results in the production of approximately 4 m^3 of slurry waste (Mikula et al., 2008). As of 2002, approximately 120 million barrels of sweet crude oil were produced annually from the Athabasca oil sands industry. This annual production is expected to rise to 400 million barrels per year as conventional sources of petroleum deplete and greater demand is placed on more unconventional oil sources such as the oil sands (Holowenko et al., 2002). The combination of increasing demand for oil and a zero

discharge policy for OSPW has increased the urgency to develop methods to remediate OSPW.

Previous research has demonstrated that plants can take up small organic compounds and weak acid herbicides (Rigitano et al., 1987; Doucette et al., 2005). In some cases, plants are capable of metabolizing organic compounds to a form that is no longer toxic (e.g. Lafferty Doty et al., 2003; Arthur et al., 2005). Here hydroponic experiments were conducted with three different native emergent aquatic macrophytes [cattail (*Typha latifolia*); common reed grass (*Phragmites australis*); and hard stem bulrush (*Scirpus acutus*)] to determine if these plants enhanced the dissipation of an oil sands NA mixture from a hydroponic system. As well, the phytotoxicity of NAs to aquatic macrophytes was monitored as to date there has been minimal research conducted on the specific toxic effects of oil sands NAs in aquatic plants. Hydroponic experiments were conducted with NAs predominantly as: ionized (medium pH = 7.8), or non-ionized (medium pH = 5.0), to determine if either dissipation or phytotoxicity is affected by the chemical form of the NAs. Finally, a toxicity investigation was conducted on hydroponic medium pre- and post-phytoremediation treatment to determine the efficacy of NA phytoremediation using wetland plants.

3.2 Materials and Methods

3.2.1 Chemicals and materials

Unless otherwise noted, all chemicals and materials used in the growth chamber experiment, sample clean up, and analysis were obtained from Fisher Scientific (Edmonton, AB, Canada).

3.2.2 Oil sands naphthenic acid extract preparation

The methods used to prepare and quantify the oil sands NA extract used in the present study are described in full elsewhere (Section 2.2; Armstrong et al., 2008). Briefly, the extract was prepared using an adapted liquid-liquid extraction method

describe previously (Rogers et al., 2002a; Janfada et al., 2006) from OSPW collected in July 2005 from an oil sands extraction operation (Fort McMurray, AB, Canada). The extract had a final NA concentration of 6,800 mg L^{-1} in 0.1 N KOH adjusted with 18.76M H_2SO_4 to a final pH of 8.0.

3.2.3 Hydroponic experiments

Plants were obtained as root cuttings from a native wetland plant nursery (Bearberry Creek Water Gardens, Sundre, AB, Canada) in October 2005 for the ionized NA experiments in medium pH = 7.8 and in June 2006 for non-ionized NA experiments in medium pH = 5.0. Root cuttings were mass cultured for at least three weeks in plastic containers with quarter-strength modified Hoagland's nutrient medium (Armstrong et al., 2008) in an environmental chamber. The environmental chamber was maintained at a 16:8 hour, 22^{o}C:18^{o}C day/night cycle, an average light intensity of 12,000 lux, and an average relative humidity of 30%:35% for day and night respectively. Once the plants were acclimatized to the chamber conditions, plants that were ~62 cm in shoot length, 7 cm long rhizome, and ~25.7 g fresh weight (cattail); ~48 cm in shoot length and ~5.6 g fresh weight (common reed); and ~66 cm in shoot length and ~15.0 g fresh weight (bulrush) were set up for two weeks prior to the start of the experiment. Plants were grown in a hydroponic system similar to the apparatus described by Doucette et al. (2005) except that 2.5 L amber borosilicate glass jars were used instead and the system was aerated with the use of aquarium air pumps (Figure 2.1). Planted treatments contained plants in the hydroponic testing system; where as unplanted treatments were not planted and were composed only of hydroponic medium in the hydroponic testing system. Each planted treatment contained three plant replicates (one replicated is equal to one plant in an individual testing system) and there were three different NA treatments (0 mg L^{-1} NAs; 30 mg L^{-1} NAs; and 60 mg L^{-1} NAs) applied for each species investigated. The treatment doses were based on the results of preliminary experiments with cattail and a commercial NA mixture (Fluka, Sigma-Aldrich, Ltd.; data not shown). A dose of 60 mg L^{-1} caused acute toxicity in the preliminary trials whereas 30 mg L^{-1} was the lowest observed adverse effects level (LOAEL). Two treatments were dosed with 60 mg L^{-1} and

served as unplanted controls. One of these unplanted controls was sterilized with 1050 mg L^{-1} sodium azide to monitor abiotic loses of NAs to the hydroponic system (abiotic control group). The other unplanted control was used to account for biotic loses due to microbiological communities that likely form in the hydroponic medium over the course of the experiment (biotic control group). A closed-cell plastic foam plug which fitted snug to the opening of the test vessel was used to support the plants and to keep the evaporation of hydroponic medium to a minimum. Transpired hydroponic medium was monitored by measuring water uptake (Trapp et al., 2000) and topped up every 5 days. The pH of the medium was maintained at either 7.8 or 5.0 using 1.0 N HCl or KOH. On Day 0, immediately after spiking with NAs and adjusting the pH, a 2 mL hydroponic medium sample was collected. The sample was collected using a pasteur pipette while the medium was stirred with a magnetic stir bar. Hydroponic medium samples were taken again on Days 5, 10, 20 and 30. Plant fresh weights were recorded on Days 0 and 30. On the appropriate day (either Day 0 or Day 30) the systems were removed of plants (in the case of planted Day 30 treatments) and ~500 mL of the medium was poured out of the test system for future testing. The remaining ~2 L in the test system was shipped (in the same 2.5 L amber glass testing vessel) to ALS Laboratories (Winnipeg, MB, Canada) for the *Daphnia magna* acute toxicity testing. All acute toxicity tests were conducted within five days of sampling.

3.2.4 Analysis

Hydroponic medium samples were analyzed for NAs using a Quattro Ultima (Waters Corp. Milford, MA, USA) triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface operating in the negative ion mode. Instrument operating parameters are reported elsewhere (McMartin et al., 2004; Appendix A). Preliminary studies found that hydroponic medium samples could not be directly injected for accurate analysis due to background ion interference (Appendix B). As a result, samples were cleaned up prior to analysis, using ENV+ solid phase extraction (SPE) cartridges (Biotage, Chartlottesville, VA, USA) (Headley et al., 2002). Using a SPE procedure described in a previous study (Section 2.3.4; Armstrong et al., 2008) the

resulting SPE extract for a given 1.5 mL hydroponic medium sample was concentrated to a final extract volume of 1 mL in 50:50 acetonitrile:water and 0.1% NH_4OH.

3.2.5 Selective uptake

In addition to measurements of total NA dissipation, an investigation was conducted to determine if plants selectively enhance the dissipation of individual NA compounds from the total NA mixture. The relative abundance of each NA within the mixture was determined by arranging the mass spectrum data according to carbon number and Z family. This method of analysis was modeled after Holowenko and coworkers (2002).

3.2.6 *Daphnia magna* LC_{50} acute toxicity tests

The *Daphnia magna* LC_{50} toxicity tests were static, acute, 48-hour tests performed by ALS Laboratories (Winnipeg, MB, Canada) following the Environment Canada (2000) reference method. Specifically, 200 mL volume is used in each test with 10 neonates allocated per test (20 mL per neonate). The tests were carried out in plastic cups such that the final solution depth was 7 cm. The samples were aerated prior to the start of the test but remain unaltered other than the appropriate dilutions for determining the LC_{50} for the duration of the 48 hour test.

3.2.7 Data analysis

Data were tested for normality and homogeneity of the variances using 1-Sample Kolomogorov-Smirnov (K-S) test and Levene's tests respectively. Data that did not meet the requirements of normality and homogeneity of the variances was log-transformed. The analysis of NA treatment and chemical form (ionized vs. non-ionized) on plant growth was separated into two one-way ANOVAs because pH of the media had a significant effect on the growth of the control treatments. For ionized NAs, data were tested using a two-way analysis of variances (ANOVA) with species and NA dose as factors, and fresh weight gain as the dependent variable. Specific differences between

treatment means were determined using a Tukey's test for post-hoc analysis. For non-ionized NAs the data contained several zero values therefore one way t-tests were conducted within each species comparing the treatments from the control growth. Differences between transpiration (the dependent variable) of the different species (the factor) within the control treatments on Day 30 were tested using a one-way ANOVA. For each species and chemical form of NAs, to determine dissipation of NAs in planted treatments, sampling day was used as a factor and NA concentration the dependent variable. For each species and chemical form of NAs, to test for the effect of planted treatments on NA dissipation on Day 30, treatment was used as a factor and NA concentration was used as the dependent variable. Significant differences between the distributions of the percent abundance of carbon numbers within specific Z families over the course of the experiment were determined using a K-S test for two distributions. The LC_{50} values were calculated using the Spearman-Kärber method. Because of the loss of replicates in the ionized NA treatments in the acute toxicity testing with *D. magna*, the non-ionized NAs were analyzed using ANOVA with treatment as the factor and LC_{50}(%) as the dependent variable with post hoc analysis using a Tukey's test. All statistical analyses were carried out using SPSS 13.0 (SPSS Inc., Chicago, IL, 2004). All graphs were created using SigmaPlot 8.1 software (SPSS Inc., Chicago, IL, 2002).

3.3 Results

Naphthenic acids appeared to significantly affect plant growth in both the low dose (30 mg L^{-1}) and high dose (60 mg L^{-1}) treatments, compared to controls, in their non-ionized form (medium pH = 5.0) (Figure 3.1b). All three replicates in the high dose treatments of cattail and both the low dose and high dose treatments of common reed died after 10 days. This mortality is also apparent in Figure 3.2d and 3.2e where the transpiration of the plants is recorded over the 30 day experiment. The effect of NAs in their ionized form did not appear to have the same magnitude of effect on growth and transpiration as their non-ionized form (Figure 3.1a, Figure 3.2a – 3.2c). The only significant difference that was observed between all the ionized NA treatments and the control, was for common reed in the high dose treatment (Figure 3.1a, 3.2b). With

regards to growth between the three different species, unexposed to NAs, cattail had almost two to three times the growth over the 30 day experiment compared to common reed and bulrush (Figure 3.1). However when water uptake was normalized to starting fresh weight to minimize the variability in water uptake amongst replicates within a treatment, common reed appears to take in more water per gram of starting fresh weight than either cattail or bulrush on Day 30 in the control plants (Figure 3.2).

For predominantly ionized NAs (medium pH = 7.8) there was no significant difference in total NA dissipation observed between the planted high dose treatments and unplanted NA treatments (Figures 3.3a – 3.3c). Additionally there was no difference in dissipation observed between the three different emergent aquatic macrophyte species tested. Moreover, there was no significant effect of plant species on the dissipation of non- ionized NAs in the hydroponic systems (Figures 3.3d – 3.3f). The pattern of NA dissipation over the course of the 30 day experiment in all three species tested appeared to be only different between the ionized and non-ionized treatments. In the ionized treatments the NA concentration appeared to increase by approximately 6 mg L^{-1} from Day 0 to Day 5 and then plateau at approximately 55 mg L^{-1} for the remainder of the 30 day experiment (Figures 3.3a – 3.3c). In the non-ionized treatments the starting concentration of NAs was approximately 50 mg L^{-1} and then gradually decreased over the course of the experiment to a final concentration of approximately 46 mg L^{-1} (Figures 3.3d – 3.3f). As well, the non-ionized, high dose, planted treatments also followed the trend of the two unplanted biotic and abiotic controls (Figure 3.3d – 3.3f).

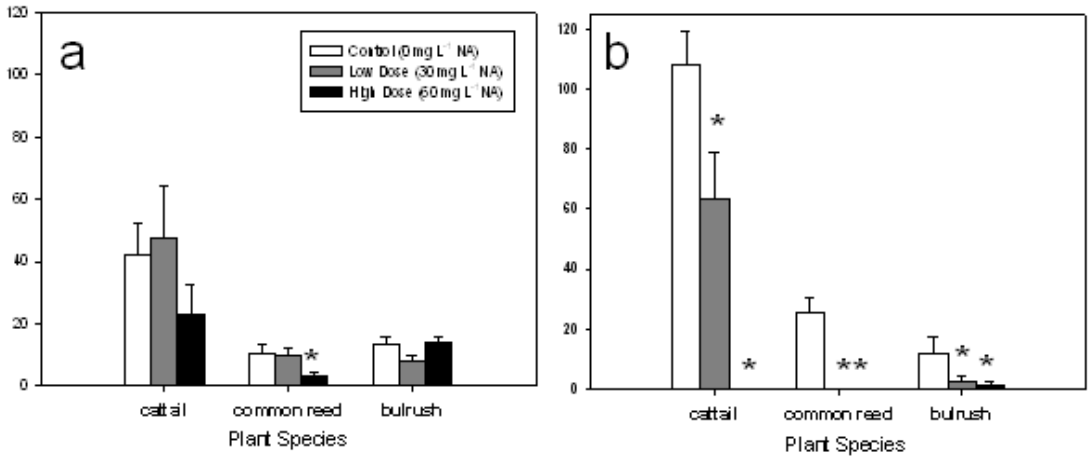

Figure 3.1: Fresh weight gain (g) in three wetland plant species (cattail, common reed, and bulrush) in different oil sands naphthenic acids (NAs) treatments with the hydroponic media at a) pH = 7.8 (ionized NAs) and b) pH = 5.0 (non-ionized NAs) over 30 days. Values represent the mean (n = 3) plus and minus one standard error. Significant differences from the control group are indicated with an asterisk (*) $P < 0.05$.

More subtle changes in certain groups of NAs over the course of the experiment become evident for plots of the total NA concentrations in the three dimensional format of carbon number (n), Z family, and percent abundance. In Figures 3.4a and 3.4b, the percent abundance of each specific NA compound (each carbon number and Z family combination), in the cattail treatments exposed to ionized NAs does not appear to change between the start and end of the experiment. However, this is in contrast to the non-ionized treatments using the same species, cattail (Figures 3.4c and 3.4d). In this situation there is an approximately 0.81% - 0.95% decrease in total abundance in the Z = -4 (two ring NA) and -6 (three ring NA) families respectively with carbon numbers ranging between 13 and 15 (Figures 3.4c and 3.4d). The decrease in abundance of these two Z families is accompanied by an increase in abundance of approximately 0.57% of the Z = -2 (one ring NA) family with carbon numbers between 15 and 18 and an increase 0.28% of the Z = -12 (six ring NA) family with carbon numbers ranging between 18 and 20 (Figures 3.4c and 3.4d). Here Day 5 concentrations are compared with Day 30 results

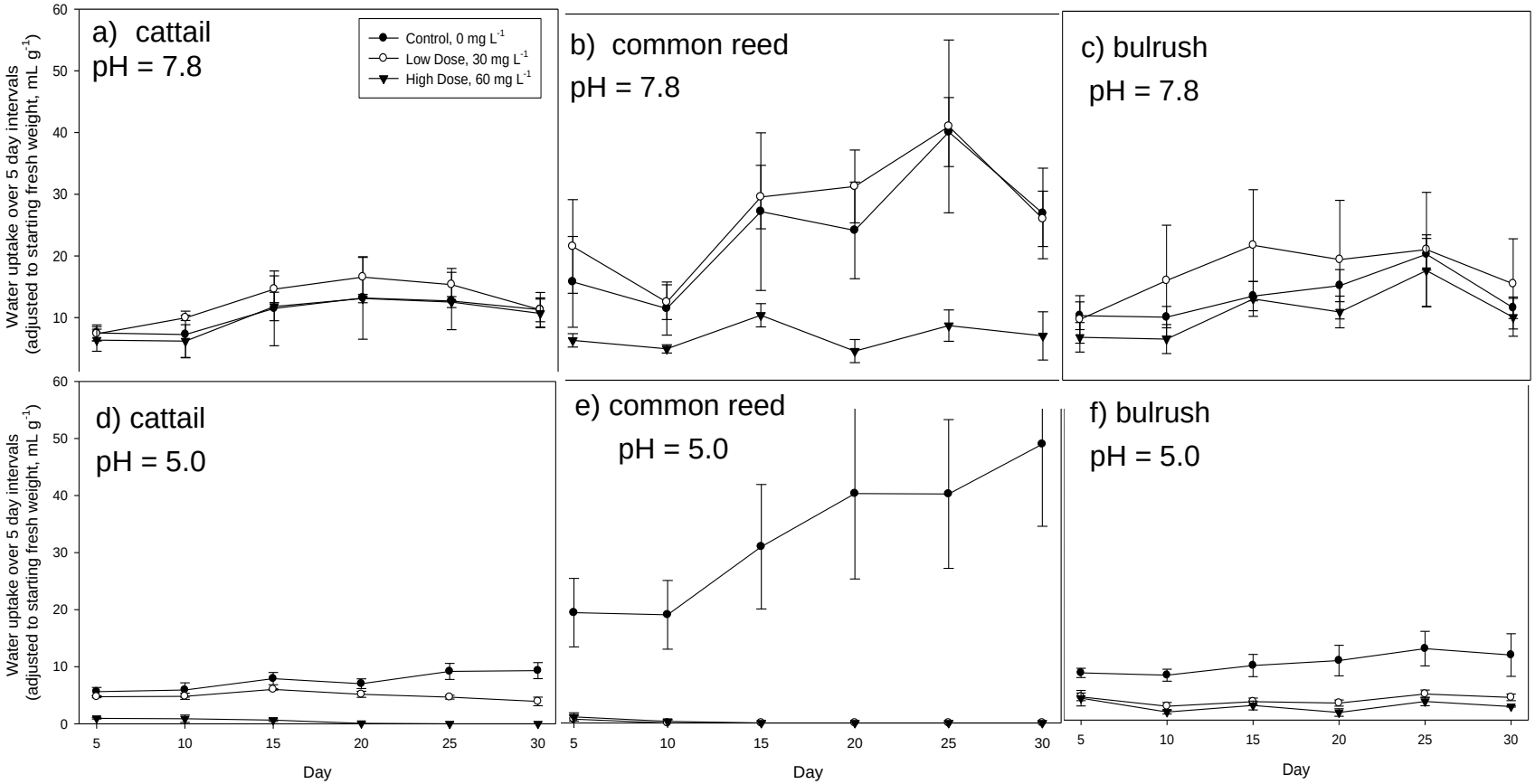

Figure 3.2: Water uptake over five day intervals (adjusted to plant starting fresh weight, mL g^{-1}) in hydroponic treatments (a – c = ionized naphthenic acids; d-f = non-ionized naphthenic acids) for three different species (a,d = cattail; b,e = common reed; c,f = bulrush). Values represent the mean (n = 3) plus and minus one standard error

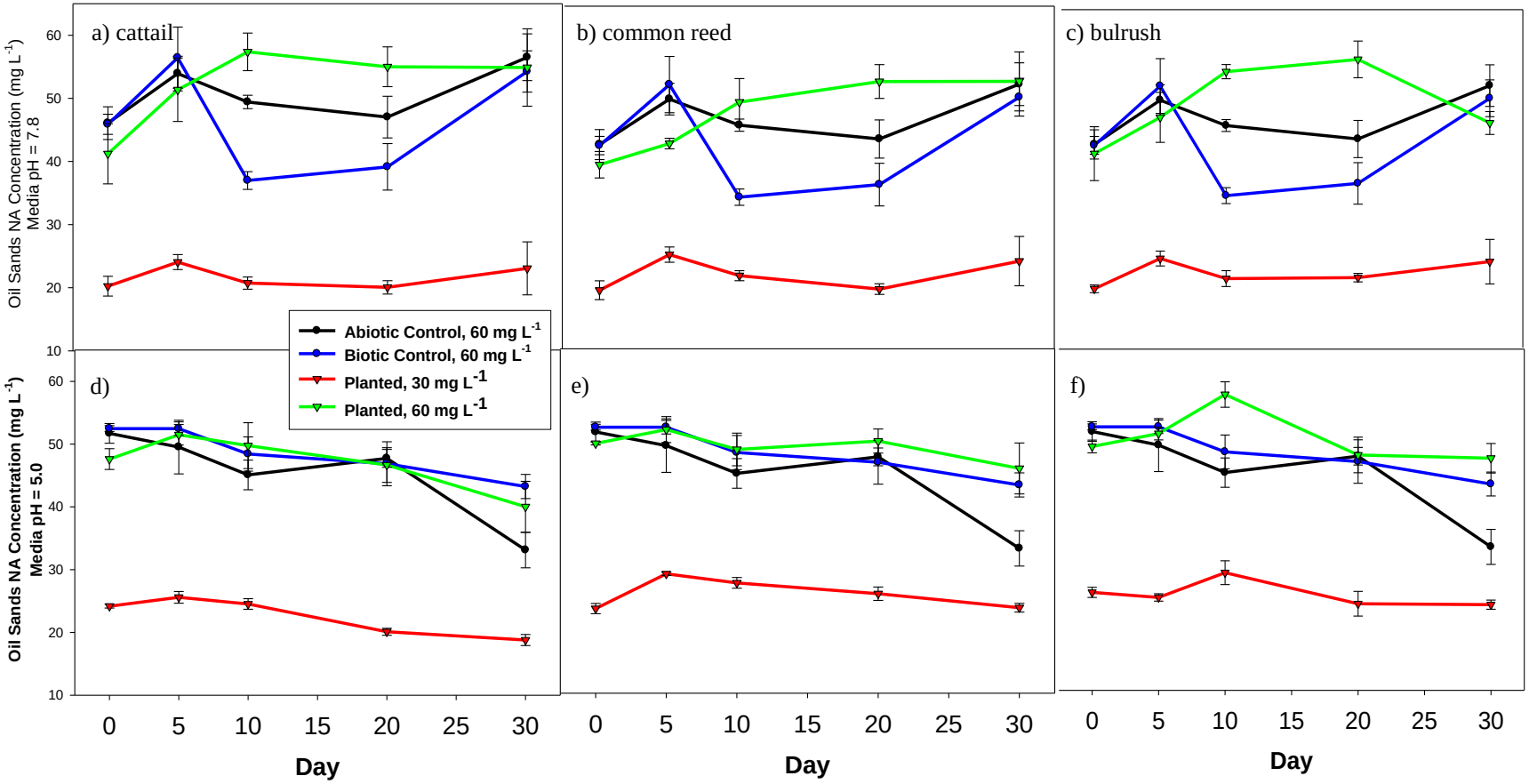

Figure 3.3: Mean (n = 3, ± SE) total naphthenic acid concentration (mg L^{-1}) in hydroponic medium over the 30 days for ionized naphthenic acids in a) cattail; b) common reed; c) bulrush and for non-ionized naphthenic acids in d) cattail; e) common reed; f) bulrush.

rather than Day 0 vs. Day 30 because the highest concentration of NAs was found on Day 5. The peak concentration being observed on Day 5 is likely a result of the NAs establishing a steady state with the hydroponic medium (Section 2.0; Armstrong et al., 2008).

To determine if this shift in NA abundance over the course of the 30 day experiment was the result of the aquatic macrophytes, the percent abundance within the two *Z* families that experienced the greatest effect over the experiment (Z = -2 and -4) were normalized and compared to unsterilized, unplanted controls (biotic controls) with 60 mg L^{-1} NAs (Figures 3.5a – 3.5d). There is a significant difference ($P < 0.05$) in the distribution of the percent abundance of carbon numbers from the start of the experiment (max abundance at C 17) to the end of the experiment (Day 30; max abundance at C 14) for both Z = -2 and -4 families in the planted treatments (Figure 3.5c and 3.5d). There is a small increase in abundance in C >14 for the Z = -4 family in the unplanted control (Figure 3.5b), however the distributions in both the Z = -2 and Z = -4 were not found to be significantly different ($P > 0.05$) between Day 5 and Day 30 (Figure 3.5a and 3.5b).

Figure 3.6 displays the mean results of the LC_{50} (%) of these NA-spiked hydroponic medium samples on either Day 0 or Day 30, Planted with the emergent macrophyte cattail or unplanted as well as the two different chemical forms of NAs (ionized or non- ionized). In the non-ionized NA treatments (pH = 5.0) there is a significant, 34% increase in the mean LC_{50} (%), which translates into a decrease in acute toxicity, from Day 0 to Day 30 (Figure 3.6). Likewise, there is a further 11% significant increase in the mean LC_{50} (%) detected from the unplanted treatments to the planted treatments on Day 30.

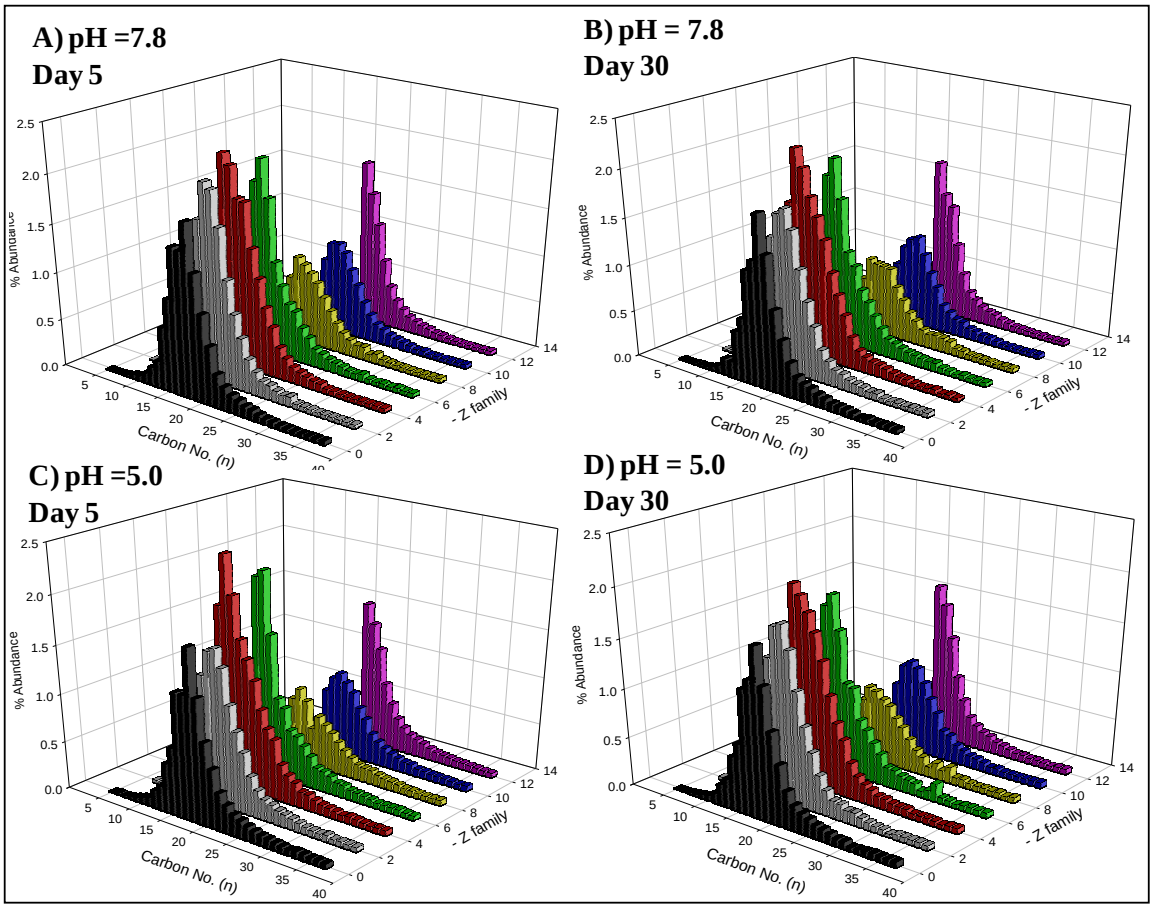

Figure 3.4: Percent abundance of ionized naphthenic acids (NAs) versus carbon number and *Z* family on a) Day 5 and b) Day 30 in cattail treatments with 60 mg L^{-1} NAs and on c) Day 5 and d) Day 30 for cattail treatments in 60 mg L^{-1} non-ionized NAs. Values are reported as an average of 3 replicates however error bars are not reported here to permit visual inspection of the data.

For the ionized NA treatments (pH = 7.8) there is more variability within the treatments (as indicated by larger standard deviation) and there is no detectable difference in LC_{50} (%) after 30 days in the unplanted treatments. In the ionized NA, planted treatment, there appears to be a significant increase in LC_{50} (%) from both the Day 0 treatments and Day 30 unplanted treatments. However because one of the replicate treatments was lost

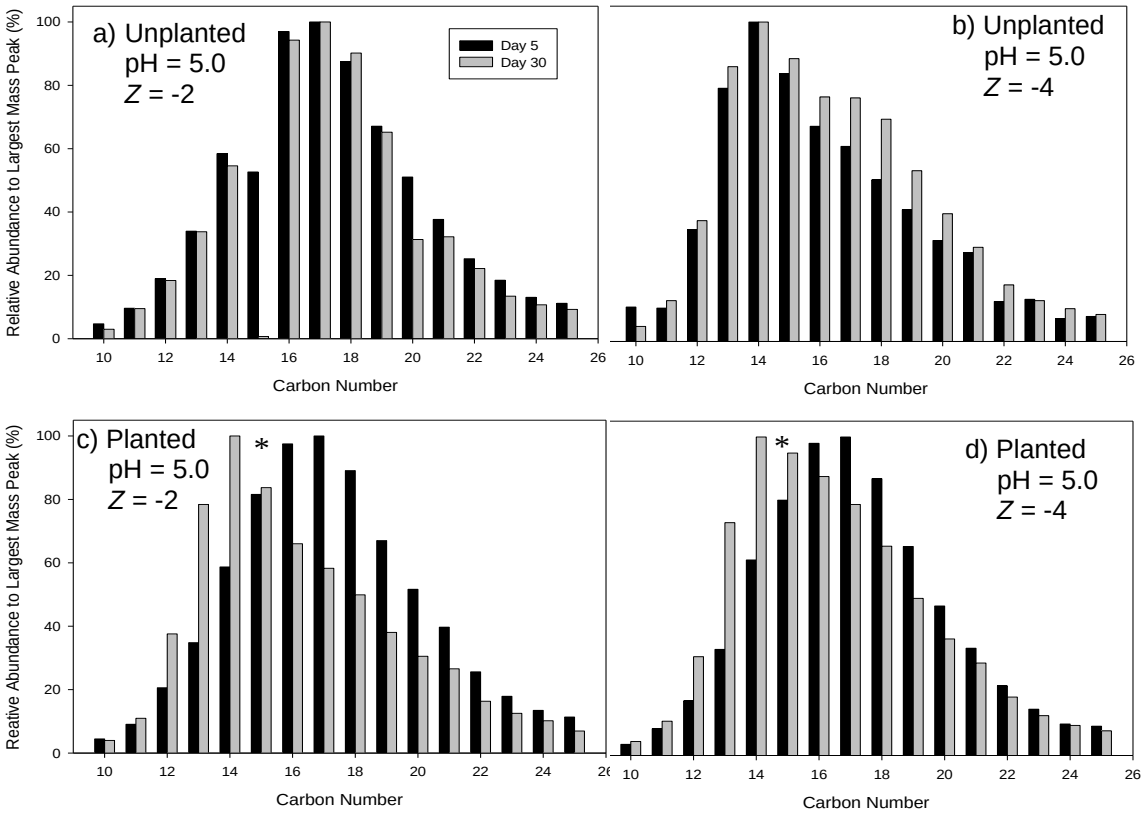

Figure 3.5: Carbon number versus the mean (n = 3) percent relative abundance of the largest mass spectra peak of the sample for unplanted biotic controls, treated with 60 mg L^{-1} non-ionized naphthenic acids, for the a) Z = -2 and the b) Z = -4 families and for the treatments planted with cattail, treated with 60 mg L^{-1} non-ionized naphthenic acids, for the c) Z = -2 and d) Z = -4 families. Significant differences in distribution (P < 0.05) on Day 30 from Day 5 are indicated with an asterisk (*).

during toxicity testing the exact LC_{50} value for this replicate could not be determined. Nevertheless, no mortality was observed when NAs were in their ionized form after 30 days of phytoremediation treatment. There was a 37% and 45% reduction in acute toxicity from the Day 0 and unplanted Day 30 treatments, respectively.

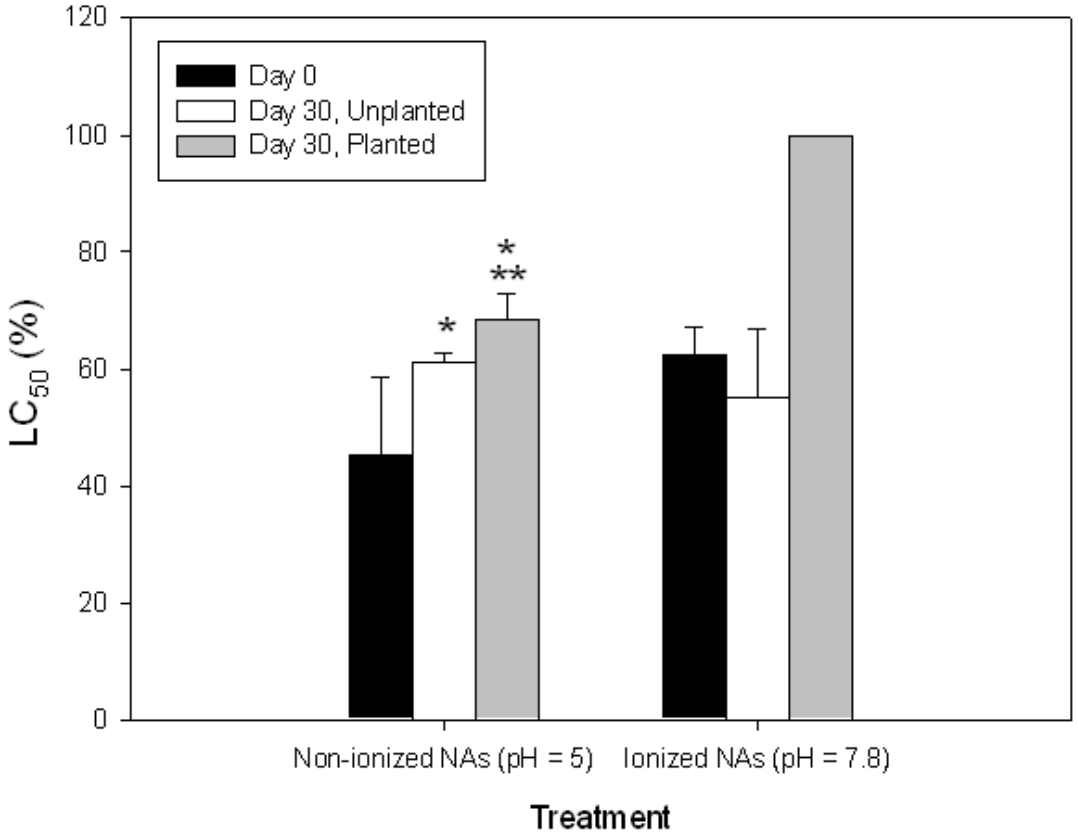

Figure 3.6: Mean LC_{50} value (%) obtained in static, acute, 48-hour, *Daphnia magna* toxicity tests. The values are reported as the mean (n = 3) ± standard deviation of the three individual testing solution replicates (i.e. the replicates from the hydroponic experiments). Significant differences between Day 0 and Day 30 are indicated with an asterisk (* ; $P < 0.05$) and significant differences between planted and unplanted treatments on Day 30 are indicated with two asterisks (** ; $P < 0.05$). The planted, Day 30 samples only had n = 2 and had $LC_{50} > 100\%$ and was therefore not included in the statistical analysis.

3.4 Discussion

The present study demonstrated that oil sands NAs appear to be more phytotoxic and likely more bioavailable to emergent wetland plants in their non-ionized form (pH < 6.0). This phytotoxic effect was noticeable within five days after exposure at 60 mg L^{-1} NAs; a concentration lower than what has been found previously in wetlands established in oil sands tailings under field conditions (i.e. NAs present in their ionized form) (Crowe et al., 2001). The effect of decreased water uptake in wetland plants observed in the present work is consistent with findings reported by others for the terrestrial plant species, jack pine (*Pinus banksiana*) exposed to a commercial NA mixture (Apostol et al., 2004). The observation that phytotoxicity increased significantly after ionization of NAs may indicate that NAs are sequestered by an "ion-trap" mechanism. In this scenario, the NAs in their lipid-soluble form are absorbed into the plant roots and then become ionized and trapped upon reaching the more alkaline phloem (Rigitano et al., 1987; Briggs et al., 1987; Riederer, 2005). Therefore, factors that lead to the lower of pH in tailings ponds would result in the OSPW being more phytotoxic.

In general, the dissipation of non-ionized NAs seemed to be similar to that of ionized NAs in the presence of wetland plants (Figure 3.3). Dissipation was found species dependent, with all three plant species observed to have similar NA dissipation rates in the hydroponic medium. For the non-ionized treatments there appeared to be a decrease in abundance of two and three ring NAs over the course of the 30-day experiment accompanied by an increase in abundance in one and six ring NAs. This change in abundance of specific NA families was found to be specific to the aquatic macrophyte treatments.

The acute toxicity studies with *Daphnia magna* here suggest that observed shifts in the molecular distribution of NAs in planted treatments after 30 days (Figures 3.5 and 3.6) significantly reduces the toxicity of the NA treated medium. In the unplanted non-ionized treatments there was also some decrease in toxicity between Day 0 and Day 30 in *D. magna*, which could be a result of microbial communities that establish in the media over the course of the experiment or due to sorption to the test vessel. Nevertheless, there is greater toxicity reduction over 30 days in the planted treatment versus the unplanted

treatment. At this time it is not possible to discern how plants are reducing the toxicity of NAs in hydroponic treatments versus their unplanted counterparts. The toxicity reduction could be from the microbial community that plants host within their roots biotransforming NAs (Arthur et al., 2005). Likewise, owing to the shift of NA molecular fingerprint observed, the plants could be storing some components or biotransforming the NAs (Scott et al., 2005). Finally, the toxicity reduction could be an indirect effect in that plant root exudates released into the hydroponic medium indirectly improve the water quality of the hydroponic medium for *D. magna*. Dakora and Phillips (2002) report a list of over 73 different compounds (made up of amino acids, organic acids, sugars, vitamins, purines, enzymes, and inorganic ions and gaseous molecules) that plants can exude from their roots for plant-plant communication, nutrient assimilation, and chemo-attraction of rhizosphere bacteria. Future research based on direct measurements of NAs in plant tissue is expected to shed light on the mechanisms of NA toxicity reduction by plants (Section 5). Nevertheless, the presence of plants is necessary in all the previously mentioned scenarios for either a direct or indirect effect on toxicity reduction.

When comparing the acute toxicity of *D. magna* in non-ionized and the ionized NA medium on Day 0 there was no significant difference observed between the two treatments in LC_{50} (%). This is surprising as in plants there is a very large significant difference in effects observed between these two chemical forms of NAs in plants with regards to fresh weight gain over 30 days (Figure 3.1). This could be a result of the plants' need to maintain turgor pressure through continual uptake and evapotranspiration of water (Trapp et al., 2000) thereby increasing the chances of uptake and exposure of NAs (Riederer, 2005). This observation provides further indirect evidence of NA uptake into plants.

Despite the phytotoxicity observed in plants hydroponically exposed to non-ionized NAs, some of the planted treatments were able to recover by sending out new shoots from the rhizome (Appendix C). Therefore it is possible that after establishment of a treatment wetland containing emergent macrophytes there may be regeneration of the system after diebacks as a result of NAs exposure. This re-growth of plant tissue after contaminant exposure and subsequent dieback has been reported previously (Delaune et al., 2003). Here, the authors reported a greater recovery from oiling by crude oil in the

field sites versus plants in a greenhouse study in a variety of wetland plant species (including cattail). Delaune et al. (2003) attributed the regeneration in field studies to be a result of having a more extensive root system in the field and because the roots are major storage sites for carbohydrates and thus the plant could recover.

It is also unknown whether or not plant cells are able to metabolize NAs in plant tissue. Knowledge of this is of significance if there are plant die-offs (e.g. during the winter) as unmetabolized NAs in plants could be re-released back into the environment during the decomposition. Rogers et al (2002b) studied the oral toxicity of NAs in rats and found increased liver weights in exposed individuals and attributed this possibly to the increased production of the detoxifying enzymes cytochrome P450. Plants do not contain detoxifying organs like the mammalian liver; however, they do produce a variety of cytochrome P450 enzymes in their cells which could be induced to metabolize environmental contaminants (Durst, 1991; Sanderman, 1994) and warrants further study.

3.5 Conclusions

In summary, it was demonstrated that NAs appear to be more phytotoxic in their non-ionized form (pH < 6). There is evidence that wetland plants are either directly or indirectly responsible for selective NA dissipation when exposed to NAs in their non-ionized form. Although NAs are predominantly found in their ionized form in OSPW, the findings of this study are important to consider for long term management of the tailings ponds. Emergent macrophytes were able to reduce the acute toxicity of NA treatments to *D. magna* after 30 days by ~34% and 37% in non-ionized (pH = 5.0) and ionized NA (pH = 7.8) treatments respectively. These reductions were approximately 11% greater than similar unplanted treatments for non-ionized NAs and 45% greater in the ionized NAs compared to unplanted treatments after 30 days. The mechanism through which planted systems achieve this toxicity reduction was not clearly established, and this warrants future investigation.

4.0 Relationship of Root-Associated Microorganisms and Aquatic Macrophytes in Oil Sands Naphthenic Acids and Phytoremediation

4.1 Introduction

Naphthenic acids (NAs) are classified as a group of organic acid compounds with the general chemical formula of $C_nH_{2n+z}O_2$ where n is equal to the number of carbon atoms, and z is equal to zero or a negative even number and represents the number of hydrogen atoms lost as a result of the structures becoming more compact (Fan, 1991). Naphthenic acids are found naturally in petroleum deposits and are released from oil sands during the caustic hot-water processing of bitumen for oil. As a result NAs accumulate in the large volumes of oil sands processed water (OSPW) produced at Athabasca oil sands operations in Fort McMurray, Alberta, Canada. There are estimated to be over 100 individual NA compounds found in OSPW (Lai et al., 1996; Headley and McMartin, 2004). Naphthenic acids are of concern in OSPW because they can subsequently migrate into aquatic environments (Bendell-Young et al., 2000; Crowe et al., 2001). In addition to their water solubility, NAs are also of toxicological concern in the environment because they have acute aquatic toxicity to a variety of aquatic organisms including fish (Headley and McMartin, 2004; Clemente and Fedorak, 2005).

Currently, after water recycling, about 4 m^2 of liquid tailings is produced for every 1 m^2 of oil sands mined (Mikula et al., 2008). Since the companies mining the oil sands in Alberta are held to a zero discharge policy, OSPW are subsequently stored in large tailings ponds (Holowendo et al., 2000). Thus, there is a need to develop methods to remediate NAs from OSPW (Quagraine et al., 2005). One of the remediation strategies currently under investigation is the use of phytoremediation with emergent aquatic macrophytes in constructed treatment wetlands (Armstrong et al., 2008).

Phytoremediation, the use of plants to reduce contamination, relies on the synergistic relationship between plants and their root-associated microbial communities, with both the plant and rhizosphere bacteria playing a part in the eventual fate of

contaminants (Siciliano and Germida, 1997; Phillips et al., 2006). Previous research with NAs and the three native emergent macrophytes cattail (*Typha latifolia*), common reed (*Phragmites australis*), and hard-stem bulrush (*Scirpus acutus*), demonstrated that NAs in their ionized form are phytotoxic to plants (Armstrong et al., 2008). Increased toxicity was also noted between experiments using NAs extracted from OSPW and NA extracts available commercially (Armstrong et al., 2008; Holowenko et al., 2002; Scott et al., 2005). However, despite differences in toxicity associated with different NA extracts, little increased dissipation of total NA concentration was detectable using low resolution mass spectrometry in systems planted with emergent aquatic macrophytes (Armstrong et al., 2008). The purpose of the present study was to investigate the microbial community inhabiting the rhizosphere of the native emergent aquatic macrophyte, cattail (*Typha latifolia*), and determine if is affected by the presence of NAs. Furthermore, this study investigated whether such an effect could be correlated with any of the observed phytotoxicity observed in aquatic macrophytes after NA exposure.

The community structure of bacteria associated with the rhizosphere of cattail in different NA treatments was assessed using a variety of molecular techniques. First total community DNA was extracted from three different root associated niches; hydroponic medium, root surfaces, and root interiors. Total DNA was amplified by polymerase chain reaction (PCR) using universal eubacterial primers, and then 16S rRNA gene fragments were separated using denaturing gel gradient electrophoresis (DGGE). Banding patterns were compared using the Jaccard similarity coefficient and bands of interest were excised, sequenced, and compared to a national database. These molecular approaches provide a rapid analysis of the microbial community and provide insight into differences in microbial communities with respect to their environment (Nakatsu, 2007).

4.2 Materials and Methods

4.2.1 Chemicals and materials

Fluka commercial naphthenic acid mixture (Fluka NA) was obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Unless otherwise noted, all other chemicals and materials used in the growth chamber experiment, sample clean up, and analysis were obtained from Fisher Scientific (Edmonton, AB, Canada).

4.2.2 Oil sands naphthenic acid extract preparation

A NA extract was prepared from approximately 500 L of OSPW collected in July 2005 from an oil sands extraction operation (Fort McMurray, AB, Canada). The NA extract was prepared using a liquid-liquid extraction method originally described by Rogers et al. (2002a) and Janfada et al. (2006). Further details on the preparation and analytical verification of the NA extract used in the present study are given in Armstrong et al. (2008). The prepared oil sands extract had a final volume of ~3 L with an NA concentration of 6,800 mg L^{-1}.

4.2.3 Hydroponic experiments

Plants were obtained as root cuttings from a native wetland plant nursery (Bearberry Creek Water Gardens, Sundre, AB, Canada) in June 2005 for the commercial NA experiments and October 2005 for the oil sands NA experiments. Root cuttings were mass cultured for at least three weeks in plastic containers with ¼ strength modified Hoagland's nutrient medium (Armstrong et al., 2008) in an environmental chamber. The growing conditions were as follows: 16:8 hour, 22^{o}C:18^{o}C day/night cycle, an average light intensity of 12,000 lux, and an average relative humidity of 30%:35% for day and night respectively. Once the plants were acclimatized to the chamber conditions, plants that were ~62 cm in length, 7 cm long rhizome, and ~25.7 g fresh weight were set up for two weeks prior to the start of the experiment. Plants were grown in a hydroponic system

similar to the apparatus described by Doucette et al. (2005) except here, 2.5 L amber borosilicate glass jars covered with aluminum foil (to prevent algal growth) were used and the system was aerated with the use of aquarium air pumps. Planted treatments contained plants in the hydroponic testing system; where as unplanted treatments were not planted and were composed only of hydroponic medium in the hydroponic testing system. Each planted treatment contained three plant replicates (one replicated is equal to one plant in an individual testing system) and there were three different NA treatments (0 mg L^{-1} NAs; 30 mg L^{-1} NAs; and 60 mg L^{-1} NAs) applied on each species investigated. The doses employed were based on the results of preliminary experiments with cattail and Fluka NAs (data not shown). A dose of 60 mg L^{-1} caused acute toxicity in the preliminary trials where as 30 mg L^{-1} resulted in low levels of adverse effects. Two treatments were dosed with 60 mg L^{-1} and served as unplanted controls. One of the unplanted controls was sterilized with 1050 mg L^{-1} sodium azide to monitor abiotic loses of NAs to the hydroponic system (glass jar, air tube, etc.). The other unplanted control was used to account for biotic loses due to microbiological communities that may form in the hydroponic medium over the course of the experiment. A closed-cell plastic foam which fitted snug to the opening of the test vessel was used to support the plants and to help reduce the evaporation of hydroponic medium to a minimum. Transpired hydroponic medium was topped up every 5 days. After topping up the media every five days but prior to taking hydroponic medium samples the pH was monitored and adjusted to 7.8 using 1.0 N HCl or KOH. While being stirred with a magnetic stir bar, a 2 mL hydroponic medium sample was taken using a pasteur pipette on Day 0 immediately after spiking with NAs and then again on Days 5, 10, 20 and 30. Plant fresh weight was recorded on Days 0 and 30.

4.2.4 Naphthenic acid analysis

Hydroponic medium samples were analyzed for NAs using a Quattro Ultima (Waters Corp. Milford, MA, USA) triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface operating in the negative ion mode. Instrument operating parameters are reported elsewhere (McMartin et al., 2004; Appendix A).

Preliminary studies found that hydroponic medium samples gave high background ion interference (Appendix B). Samples were therefore cleaned up using ENV+ solid phase extraction (SPE) cartridges (Biotage, Chartlottesville, VA, USA) (Headley et al., 2002). The SPE method used for these hydroponic samples is reported elsewhere (Section 2; Armstrong et al., 2008).

4.2.5 DNA extraction for molecular analysis

Bulk, rhizoplane and endophytic communities were evaluated immediately following NA addition (Day 0) and at the end of the experiment (Day 30). To assess rhizoplane communities, a 3 – 6 g sample of roots from each replicate with attached sediment, was suspended in 5 volumes of monopotassium phosphate (MPP) buffer (0.65 g K_2HPO_4, 0.35 g KH_2PO_4, 0.10 g $MgSO_4$ in 1 L water) in a sterile erlenmeyer flask. The samples were then shaken on a rotary shaker for 1 hour, and the resulting slurry was decanted into a sterile Falcon tube. Roots were then rinsed with an additional 5 volume aliquot of MPP buffer and this rinsate was added to the rhizoplane slurry in the Falcon tubes. To assess endophytic communities, rinsed roots were surface disinfected by sequential washes with 95% ethanol and 5.25% sodium hypochlorite, followed by a minimum of 5 rinses with sterile water. To assess surface sterility, 100 µL aliquots of the final rinse water were spread on $1/10^{th}$ TSA plates. An additional 1 mL aliquot of the final wash water, boiled to release DNA, was assessed by PCR using the eubacterial primers outlined in the following sections. Roots were stored at 4^oC for 24 hours while awaiting results from sterility assessments. Endophytic extracts were subsequently produced by macerating 1 g of surface-sterile root from each treatment replicate in 9 mL MPP buffer using a sterile mortar and pestle.

Samples of all treatment replicates were stored at -20^oC until molecular analysis. Bulk water microbial communities were removed from 50 mL bulk water by filtration through a sterile 0.45 µm filter, which was then frozen. Rhizoplane slurries were centrifuged (5 min at 7600 rpm, Beckman model TJ-6 centrifuge), the resultant supernatants were filtered through a sterile 0.45 µm filter, and filters and pelleted rhizoplane sediments were combined and frozen. Macerated roots were frozen in MPP buffer.

4.2.6 Assessment of total heterotrophic communities

Medium, rhizoplane sediment and endophytic extracts were serially diluted in MPP buffer and total heterotrophic bacteria were enumerated by plating in triplicate 100 µL of each dilution (10^{-3}-10^{-7}) from each treatment on 1/10 TSA plates containing 0.1 g L^{-1} cycloheximide. Plates were incubated at room temperature for 14 days.

4.2.7 Assessment of microbial community structure

Total community DNA was extracted from all samples using a previously outlined bead-beating protocol (Phillips et al., 2006). This method used a combination of bead-beating, proteinase K, and sodium dodecyl sulphate to lyse cells. Proteins and cellular debris were precipitated using 7.5M ammonium acetate, and DNA was subsequently precipitated using isopropanol, re-suspended in 100 µL TE (pH 8.0), and purified using PVPP columns. DNA yield was quantified on ethidium bromide-stained 0.7% agarose gels by comparison with a high DNA mass ladder (Invitrogen Canada Inc., Burlington, ON, Canada).

Community structure and taxonomic diversity were examined by DGGE analysis of PCR-amplified 16S fragments. Total DNA extracts from each treatment were amplified using the universal eubacterial 16S primers U341-GC (5'-CCTACGGGAGGCAGCAGGCGGGGCGGGGCGGGGGCACGGGGGGGCGCGGCGGGCGGGGCGGGGG, (Lee et al., 1993)) and U758 (5'-CTACCAGGGTATCTAATCC, (Rölleke et al., 1996) using the PCR protocol outlined in Phillips et al. (2006). Correct PCR amplification was confirmed on ethidium bromide-stained 1.4% agarose gels. Pooled PCR reactions were precipitated with 0.1volumes of 3M sodium acetate and 2.5 volumes of 100% ethanol at -20^{o}C overnight and re-suspended in 15 µL of TE buffer (pH 8.0), and quantified on ethidium bromide-stained 1.4 % agarose gels by comparison with a low DNA mass ladder (Invitrogen Canada Inc., Burlington, ON, Canada).

DGGE was performed on a Bio-Rad DCode system (Bio-Rad, Mississauga, ON, Canada) essentially as described by Lawrence et al. (2004). For each treatment, 300 ng of amplified 16S rRNA gene product was loaded per lane onto an 8% acrylamide gel with a 40-60% urea-formamide denaturing gradient. Electrophoresis was performed for 16 h at

80V and 60^{o}C. The resulting gel was stained with SYBR Green I (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) in TAE buffer and visualized using a digital gel documentation system (GelDocMega; BioSystematica, Devon, United Kingdom).

DGGE bands of interest were excised from the gel using a scalpel and DNA was eluted in sterile deionized water by overnight incubation at 33^{o}C. DNA was re-amplified using the primer set U341 and U758 as described by Juck et al. (2000), with the addition of 6.25 µg BSA (Amersham Biosciences, NJ, USA) to each 50 µL reaction mixture. Amplification proceeded for 25 cycles of 1 min denaturation at 94^{o}C, 1 min annealing at 60^{o}C, 1 min extension at 72^{o}C, and a final extension of 3 min at 72^{o}C. Re-amplified DNA was pooled and precipitated as described above, run on a 1.0% agarose gel, and extracted and purified from the gel using the GeneClean gel extraction kit (Qbiogene, Inc., Irvine, CA, USA). Purified 16S rDNA fragments were sequenced using the ABI Prism 377 automated fluorescence sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were submitted for comparison to the GenBank databases using the BLAST algorithm (Altschul et al., 1997). Dendrograms were created by cluster analysis of the non-weighted DGGE banding patterns, using the Jaccard similarity coefficient and the UPGMA clustering method (BioNumerics software, Applied Maths, Austin, TX, USA).

Individual sample replicates were extracted and amplified using PCR and then separated using DGGE. The banding patterns of gels from individual inter-treatment replicates were compared and when it was determined that they produced comparable banding patterns, DNA from the three replicates from within each treatment was pooled. Pooled DNA was then re-amplified using PCR and then separated again using DGGE. Bands from the pooled DGGE were excised and sequenced. Several DGGE were produced from pooled DNA and dendograms were created for these replicate DGGEs and compared to determine that the stability of the DGGE banding pattern.

4.2.8 Data analysis

Data were tested for normality and homogeneity of the variances using a 1-Sample KS Test and Levene's tests respectively. Data that was not normal and

homogeneous was log-transformed. To determine the effect of NA treatment and NA mixture on plant growth a two-way analysis of variance (ANOVA) was conducted with dose and NA mixture as factors and fresh weight gain as the dependent variable. Specific differences between the treatment means were determined using a Tukey's test for post-hoc analysis. For each NA mixture, to determine dissipation of NAs in planted treatments, sampling day was used as the factor and NA concentration the dependent variable in a one-way ANOVA. For each species and NA mixture, to test for the effect of planted treatments on NA dissipation on Day 30, treatment was used as the factor and NA concentration was used as the dependent variable. All statistical analyses were carried out using SPSS 13.0 (SPSS Inc., Chicago, IL, 2004) All graphs were created using SigmaPlot 8.1 software (SPSS Inc., Chicago, IL, 2002).

4.3 Results

Naphthenic acids did not significantly affect plant growth in the low dose treatments. However, at the high dose of 60 mg L^{-1} NAs, there was a significant, 90% reduction, in plant fresh weight gain in the commercial Fluka oil sands mixture compared to the control (Figure 4.1). Oil sands NAs also impacted plant fresh weight gain however this effect was not found to be significantly different from the control group and is in part likely due to high variability within the treatments (Figure 4.1). The aqueous phase concentration of both types of NAs mixtures in the hydroponic systems appeared to peak from Day 0 to Day 5 (Figures 4.2a and 4.2b). While the NA concentration in the 60 mg L^{-1} Fluka NA treatments appeared to dissipate ~35% from Day 5 to Day 30 (Figure 4.2a) the concentration of oil sands NAs during this same period did not appear to dissipate significantly during this same period $P < 0.05$ (Figure 4.2b).

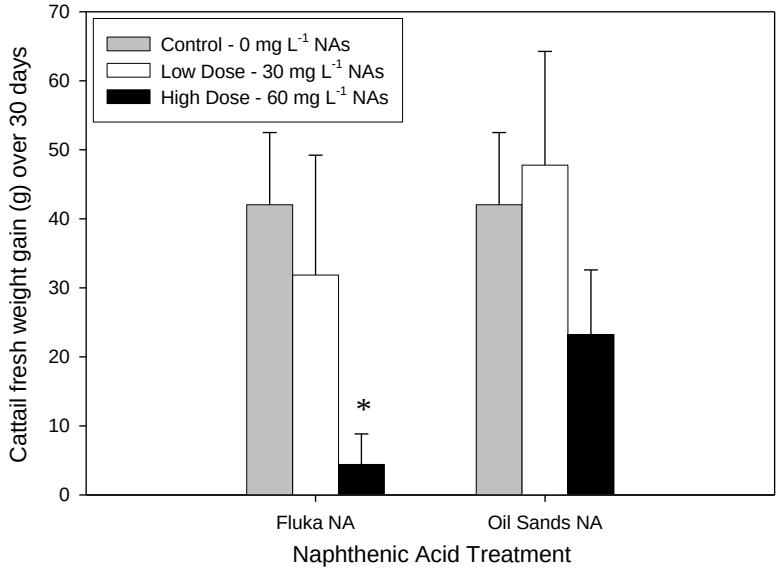

Figure 4.1: Fresh weight gain (g) in cattails over a 30 day exposure to Fluka commercial naphthenic acids (Fluka NAs) and oil sands naphthenic acids (Oil Sands NAs). Values are reported as the mean ± SE and significant differences from the control are indicated with an asterisk (*, $P < 0.05$, n = 3).

dissipation between the planted and unplanted 60 mg L^{-1} NA treatments (Figure 4.2a) Surprisingly, this trend of NA loss over time was not observed in the treatments with 30 mg L^{-1} NAs as would be expected (Figures 4.2a and 4.2b). The toxicity observed after NA exposure accompanied by no significant NA dissipation in planted treatments supports the hypothesis that rhizosphere bacteria could be playing a role in the phytotoxicity of NAs.

Bulk water and rhizosphere bacteria increased by an order of magnitude over the course of the experiment, from 2 x 10^5 to 2 x 10^6 CFUs mL^{-1} water and from 1 x 10^9 to 1 x 10^{10} CFUs g^{-1} rhizoplane biofilm, respectively. In contrast the endophytic heterotrophic populations remained constant at approximately 1 x 10^7 CFUs g^{-1} of root. High doses of Fluka NAs had a negative impact on bulk water populations, resulting in significantly

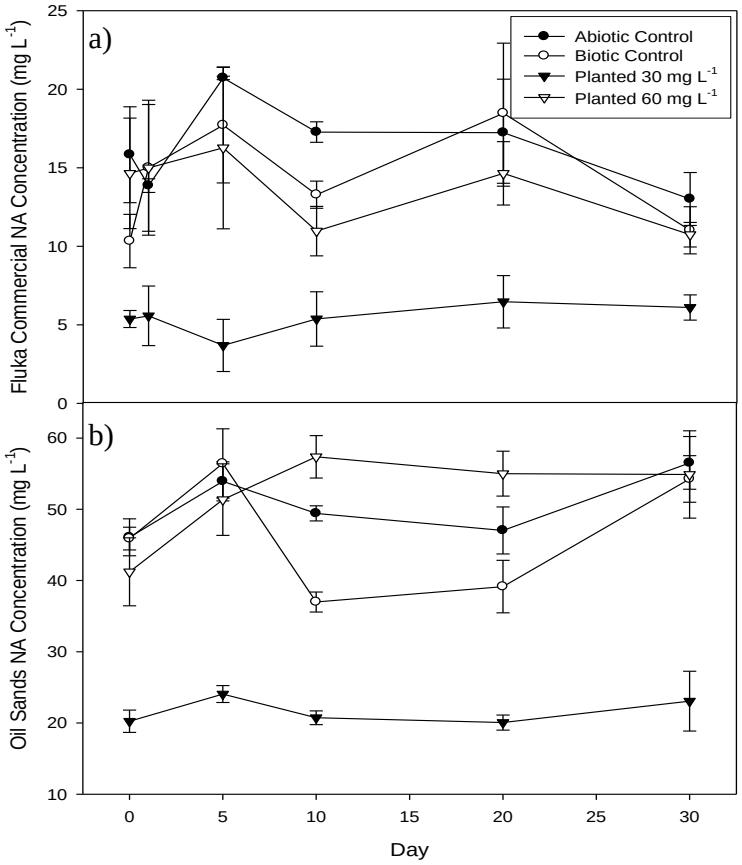

Figure 4.2: Naphthenic acid (NA) concentration (mg L^{-1}) in hydroponic media over 30 days for a) Fluka commercial NAs and b) oil sands NA extract.

lower CFUs (7 x 10^5 CFUs mL^{-1}, $P < 0.05$) than other bulk water communities. There was no significant difference in heterotrophic community numbers in either the rhizoplane or endophytic niches of different treatments.

Table 4.1 contains a summary of identified DGGE bands with interpretations of relative changes over the course of the 30 day experiment. In general, there is a decrease in community complexity observed when moving from the bulk water, rhizoplane, to the endophytic communities by Day 30 (Table 4.1). Table 4.1 and Figure 4.3 show that from the bulk water to the endophytic bacterial community (that is from the outside to the inside of the plant root); there is a decreasing effect of NAs on community structure. This is evident by the minimal difference in banding patterns between the endophytic communities of the control and the NA treatments on Day 30 (Figure 4.3).

Another pattern that emerges from Table 1 and the DGGE in Figure 4.3 is the loss of some bacteria in response to NAs and increase in other bacteria species as a response to NAs. Figure 4.3 shows a disappearance of Band # 2 in the NA treatments compared to control treatments. This band was identified as having 100% similarity to *Sulfurospirillum* sp. originally found in chlorinated ethane-degrading cultures (Table 4.1) (Duhamel and Edwards, 2006). In contrast, Band # 16 in Figure 4.3, shows the increased proliferation of a species in response to NA exposure which is 99% similar to a *Sphingomonas* sp. identified on the NCBI database (accession number DQ010645) as being from diethyl phthalate degraders from sewage sludge (Table 4.1).

Grouping the DGGE according to the percent similarity among DGGE banding patterns using Jaccard analysis resulted in the image displayed in Figure 4.3**.** Here there is a distinct clustering of the endophytic bacterial community from the bulk water and rhizoplane bacterial communities. Within this endophytic cluster the NA treated communities are only 50% similar to the control at time 0 signifying that the endophytic community changes in plant roots with time and by the end of the experiment are 70 to 80% similar (Figure 4.3). Plants exposed to higher dosed NAs, and more phytotoxic Fluka commercial NA mixture, clustered together around 70% similarity and those plants in the control and time zero samples are grouped together around 60 to 70% similarity (Figure 4.3).

Table 4.1 lists the closest match found on the NCBI database to the 24 bands of interest excised from the DGGE. DNA extracted from the plants in this study were 96 – 100% matches to bacteria previously found either in wetland environments or in association with macrophytes (Bands # 1, 4, 8, 14, 15, 18, and 23). In general there was an increase in potentially pathogenic bacteria such as *Agrobacterium* spp. (Bands # 8 – 10) and *Dechlorospirillum* spp. (Bands # 17 and 18; Table 4.1, Figure 4.3). In contrast to this there was a decrease in relative abundance of some rhizosphere microorganisms that are considered beneficial to plant growth and found in natural freshwater habitats (Bands # 22 and # 15, respectively; Table 4.1, Figure 4.3). There was an increase in prevalence of a bacteria previously found in association with oil sands (Band # 7 and # 24; Figure 4.3). Band # 7 was 98% similar to an uncultured bacterium clone originally isolated from acidic hydrocarbon-seeping outcrop (Röling et al., 2006) where as Band # 24 was

Table 4.1: Relative change (no change, =; increase, I; decrease, D) of bacteria identified from reamplified denaturing gradient gel electrophoresis bands from different rhizosphere niches (bulk, rhizoplane, and endophytic); Naphthenic acid (NA) treatments (commercial Fluka mix, F; oil sands mix, O); NA Dose (Control, C; 30 mg L^{-1}, 1; 60 mg L^{-1}, 2); after 30 days. The source column identifies which niche bacteria appeared to originate from in the systems (E = endorhizae, R = rhizoplane, nd = not determined).

		Bulk					Rhizoplane					Endophytic					Source
Band	Probable identification*	**C**	**O1**	**O2**	**F1**	**F2**	**C**	**O1**	**O2**	**F1**	**F2**	**C**	**O1**	**O2**	**F1**	**F2**	
1	*Kaistomonas* sp. (DQ664246)	=	=	=	=	=	I	I	I	I	I	I	I	I	I	I	E
2	*Sulfurospirillum* sp. (AY780560)											I	D	D	D	D	E
3	*Novosphingobium* sp. (AJ000920)	=	I	=	I	=	=	D	D	D	=						R
4	*Mucilaginibacter paludis* (AM490402)																
5	*Azospirillum* sp. (AY129807)	I	I	I	=	=	I	I	I	=	=	=	D	D	D	D	R E
6	Uncultured bacterium clone (AJ318147)		I	I	I	I						=	=	=	=	=	E
7	Uncultured bacterium clone (AY822704)	I	I	I	=	=	I	I	I	=	D	D	D	D	D	D	R E
8	*Agrobacterium tumefaciens* strain Ar-4 (AB306893)											=	=	=	=	=	
9	*Agrobacterium* sp. strain PNS-1 (AY762361)											I	I	I	I	I	E
10	*Agrobacterium* vitis (AB247621)						I	I	I	I	I	D	D	=	=	=	E
11	*Sphingomonas* sp. HTCC399 (AY429693)	=	=	=	=	=	=	D	=	D	=						R
12	Uncultured bacterium clone:655930 (DQ404716)	=	=	=	I	I				I							nd
13	Uncultured sludge bacterium H10 (AF234693)	=	=	=	=	=	=	=	=	=	=	=	=	=	=	=	R E
14	Rhizobiales bacterium (AB174816)				I	I	=	=	I	I	I						R
15	*Polynucleobacter* sp. (AJ550671)	=	I	I	I	D	I	=	=	=	=						R
16	*Sphingomonas* sp. (DQ010645)	=	I	=	=	D	I	I	I	=	=	=	I	I	I	I	R E
17	*Dechlorospirillum* sp. (AF170352)	D	D	D	=	=	=	=	I	I	I	=	=	=	I	I	R E
18	*Dechlorospirillum* sp. (AY530551)				=	=				=	I	I	I	I	I	I	E
19	*Devosia ginsengisoli* (AB271045)		I	I	I		I	I	I			=	=	D	D	D	E
20	*Luteimonas* sp. (AB188220)	I	=	I	I	=	=	D	D	D	D						R
21	*Aquaspirillum peregrinum* (AB074521)											I	I	=	I	=	E
22	*Derxia gummosa* (AB089481)	I	=	I	I	=											nd
23	*Garrityella koreensis* (DQ665916)											=	=	=	=	=	E
24	Uncultured gamma proteobacterium (EF420207)				I	I				I	I						nd

* All sequenced bands had ≥ 95% similarity to the closest match in the GenBank NCBI database. Accession number for the NCBI database is given in brackets with the identified name.

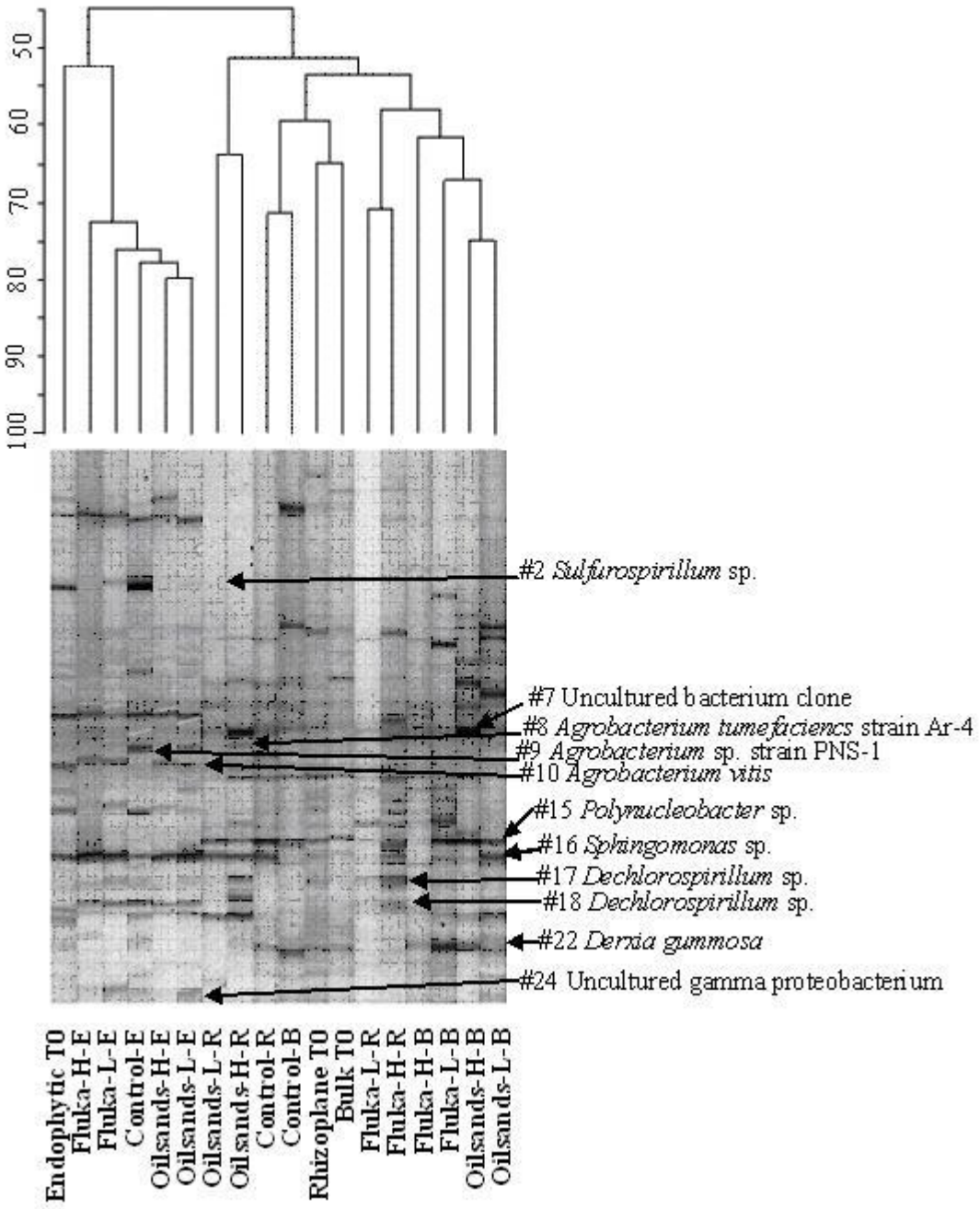

Figure 4.3: Jaccard cluster analysis of non-weighted denaturing gradient gel electrophoresis banding patterns from bulk water (B), rhizoplane (R), and endophytic (E) microbial communities of cattails growing in different doses of naphthenic acids (NAs) [Control, (0 mg L^{-1}); L, low dose (30 mg L^{-1}); H, high dose (60 mg L^{-1})], types of NAs [Fluka commercial NAs (Fluka), oil sands NAs (Oilsands)], on day 30 and day 0 (T0). Bands of interest are highlighted and identified with arrows.

identified as being 98% similar to an uncultured gamma proteobacterium clone originally found in an oil sands tailings pond in Alberta, Canada (NCBI database accession number EF420207; Table 4.1; Figure 4.3).

4.4 Discussion

The reduction in plant fresh weight gain observed in the present study has been observed in previous studies (Armstrong et al., 2008). Kamaluddin and Zwiazek (2002) observed a similar decrease in plant growth as decreased leaf size to aspen seedlings (*Populus tremuloides*) exposed hydroponically to a commercial mixture of NAs. The increased toxicity observed here in the treatments with commercial NAs has also been reported by others and is hypothesized to be a result of the low molecular weight compounds present in the commercial extract being more bioavailable (Scott et al., 2005). A comparison of the molecular fingerprint (mass chromatogram) of the total NA mixture for both commercial (Fluka) and oil sands NAs can be found in a previous study (Figure 2.6; Armstrong et al., 2008).

The differentiation of the bacteria community inhabiting the root interior (endorhiza) from the surrounding rhizosphere over time to fewer genera has been previously reported (Hallmann et al., 1997) and highlights that the endorhiza is indeed a distinct niche from the rhizosphere. The decreasing effect of NAs on community structure from the bulk water to the endophytic bacterial community (Table 4.1 and Figure 4.3) is likely because endophytic bacteria live within the plant root tissue and have lower exposure to NAs when compared to their bulk water and rhizoplane bacterial counterparts. Grouping the DGGE according to the percent similarity among DGGE banding patterns using Jaccard analysis (Figure 4.3) displays that there is a distinct clustering of the endophytic bacterial community from the bulk water and rhizoplane bacterial communities. The clustering patterns observed within the dendogram in Figure 4.3 indicates that exposure to NAs impacts the rhizosphere community that would otherwise establish in plant root systems under non-contaminated conditions. Other investigators have observed similar phenomena in plant systems with other

environmental contaminants such as petroleum hydrocarbons and salts (Phillips et al., 2006), phenoxy herbicides (Cuadrado et al., 2008), and metals (Åkerblom et al., 2007). In general there was an increase in potentially pathogenic bacteria such as *Agrobacterium* spp. (Bands # 8 – 10) and *Dechlorospirillum* spp. (Bands # 17 and 18; Table 4.1, Figure 4.3). While there is no published literature on the effects of *Dechlorospirillum* spp. on plant health, these species can reduce perchlorate to chloride ions (Waller et al., 2004) which can then interfere with plant cell osmotic regulation. In contrast to this there was a decrease in relative abundance of beneficial microorganisms (Table 4.1, Figure 4.3).

The observations made in this study appear to suggest that NAs are either toxic to beneficial microbes and/or beneficial to pathogenic microbes. By visual inspection, plants have altered root structure after 30 days exposure to NAs. Thus, NAs may affect root physiology directly; making it easier for pathogenic microbes to penetrate the plant root. In their review of bacterial endophytes, Hallmann et al. (1997) state that endophytic bacteria can colonize roots through wounds (such as those that may result from potential chemical injury from NA exposure). Direct toxicity to root surface cells may also result in the release of cell lysates which in turn would attract more pathogenic bacteria to the plant roots (Hallmann et al., 1997). Alternatively, pathogenic microbes themselves may cause the visual physiological effects observed in plant roots or NAs may be toxic to beneficial rhizosphere microbes. In these situations NA toxicity experienced by plants would be an indirect effect. The observed phytotoxicity could thus be a combination of direct and indirect NA toxicity. Parmar and Dadarwal (1999) report that in agricultural soils, soil contamination by fertilizers and pesticides has greatly affected beneficial root associated bacteria and they state that in these situations, harmful bacteria tend to predominate in the rhizosphere.

This study is unique for providing the first characterization of the rhizosphere community inhabiting the roots of aquatic plants exposed to oil sands NAs. Biryukova et al. (2007) investigated the potential for biodegradation of NAs by cultures of bacteria enriched from native terrestrial plant rhizosphere soil. However, the authors of this study did not identify the microorganisms present in the enrichment cultures nor were there any details provided as to how, if at all, the rhizosphere community extracted from native plants changes as a result of NA exposure. In addition the rhizosphere enrichment

cultures from the study were taken of only one portion of the rhizosphere community (the root surface or rhizoplane bacterial community). In the present study we investigated all three bacterial niches, bulk water, rhizoplane, and endophytic bacteria communities within plant roots. Nevertheless Biryukova and coworkers (2007) were able to demonstrate that aerobic rhizosphere enrichment cultures of native species were capable of degrading commercial NA (Merichem) extracts when present as the sole carbon source (Biryukova et al., 2007).

This investigation also identified that the cattail rhizosphere had bacteria with 98% similarity with two bacteria clones (Figure 4.3; Band # 7, unidentified; Band # 24 unidentified gamma proteobacterium) previously identified in association with acidic petroleum deposits (Röling et al., 2006) and the oil sands (NCBI database accession number EF420207) respectively (Table 4.1). Furthermore, these two species became more dominant when the NA concentration increased or when moving from the lower toxicity oil sands extract to the higher toxicity commercial NA treatment. This is most likely because the commercial NA extract is lower in molecular weight and therefore more bioavailable to microbial metabolism. This is supported by a previous study that found commercial NA extracts were more readily biodegradable than an Athabasca oil sands NA extract (Scott et al., 2005). Moreover, it is possible that under a more natural, uncontaminated rhizosphere system experience in the control systems, NA biodegradable bacteria are unable to compete with other bacteria inhabiting the same rhizosphere niche.

4.5 Conclusions

The significance of the findings presented here is that the phytotoxicity of NAs in plants is in part likely due to the effects of NAs on the rhizosphere microbial community. Therefore future investigations on the use of plants for phytoremediation of NAs will require further investigation into the development of root microbial inoculants that support plant health under NA exposure. In addition, the identification of NA-specific bacteria in the cattail rhizosphere warrants further study as to what role these bacteria may play in the degradation of oil sands NAs in planted systems.

5.0 Preliminary Investigations into Determining the Fate and Phytotoxicity of Naphthenic Acids in Plant Tissue

5.1 Introduction

Naphthenic acids (NAs) are a group of organic acid compounds which become concentrated in the oil sand processed water (OSPW) that is produced by oil sands mining operations near Fort McMurray, Alberta, Canada (Lai et al., 1996; Headley and McMartin, 2004). These compounds are known to be toxic (for a review of NA toxicity see Headley and McMartin (2004) and Clemente and Fedorak (2005)) and are recalcitrant in OSPW. Due to the toxicity of NAs, companies mining the oil sands are held to a zero discharge policy on all their OSPW. Therefore there is a need to develop strategies to remove NAs from OSPW. Phytoremediation of NAs from OSPW using emergent wetland plants and their associated root microorganisms is one remediation strategy currently under investigation (Armstrong et al., 2008).

Previous research has found that there is a change in the NA fingerprint (i.e. molecular composition determined using negative ion mass spectrometry) in hydroponic systems spiked with NAs after 30 days growth with the cattail (*Typha latifolia*) (Section 3.0). This change in the NA fingerprint in hydroponic systems which were spiked with NAs was also accompanied by phytotoxicity; evident as reduced plant growth in planted treatments of 60 mg L^{-1} NAs. However despite these observations suggesting NA uptake by cattail, there was no accompanying detection of significant dissipation of total NAs from spiked hydroponic systems using low resolution mass spectrometry (Armstrong et al., 2008). In order to better understand the remediation potential for NAs by emergent macrophytes there is a need to determine the fate of NAs directly in plant tissue. The following section reports the findings of two studies that were attempted to achieve the goal of plant tissue identification of NAs.

The first approach attempted was to extract NAs from plant tissue using more conventional analytical techniques whereby tissue was extracted and then analyzed using negative ion electrospray mass spectrometry (ESI-MS). In these experiments pressurized

liquid extraction (PLE; Dionex trade name ASE for accelerated solvent extraction) was used to extract NAs from plant tissue to produce a liquid extract for ESI-MS analysis. The use of PLE permits adjusting a variety of extraction parameters including pressure, temperature, static-times, solvents, and sample cell combinations to optimize the extraction of the analyte of interest (NAs) from solid or semi-solid matrices like plant tissue (for a review see Carabias-Martínez et al., 2005).

The second phase of this study was carried out using synchrotron fourier transform infrared (FTIR) microspectroscopy to determine if this technique could be applied to track the fate of NAs across a plant root cross-section. The advantage that this technique offers is that it can potentially discern if NAs are being adsorbed to the outside of plants roots or if they are actually taken up into plant roots where they may be available for either biotransformation to less toxic compounds, or stored in plant tissue for eventual harvest and disposal (Arthur et al., 2005). More often than not, conventional analytical techniques are destructive and would most likely be unable to have the spatial resolution necessary to determine the fate of a contaminant like NAs across a plant root cross section of less than one millimeter in diameter (Yu et al., 2003).

Synchrotron FTIR microspectroscopy has proven to be an essential tool at identifying organic molecules in complex matrices (e.g. Dokken et al., 2005; Mecozzi et al., 2007). In particular this method is proving to be well suited for the identification of the spatial and chemical composition of biomolecules within plant cells and tissues (e.g. Yu et al., 2003; Dokken et al., 2005; Pietrzak and Miller, 2005; Dokken and Davis, 2007). There are several advantages of using a synchrotron source infrared light in FTIR microspectroscopy. The increased brightness and brilliance of synchrotron light offers diffraction-limited spatial resolution thereby permitting the investigations at the cellular dimension (~3-10 μm), increased signal to noise ratios, and decreased sample acquisition times (Yu et al., 2003). Fourier transform infrared microspectroscopy is an ideal technique for toxicological studies investigating the fate of a contaminant in a tissue. In addition to tracking the fate of the chemical in a complex matrix such as tissue, information can be provided on the biochemical changes (if any) to the tissue as a result of exposure to that contaminant.

To date the exact mechanism of action of NAs in plant tissue have yet to be elucidated (Crowe et al., 2001). Kamaluddin and Zwiazek (2002) reported that a commercial NA preparation inhibited root water transport, gas exchange and leaf growth in hydroponically grown aspen (*Populus tremuloides*) seedlings. The authors suggested that NAs affected gas exchange and growth in aspen by interfering with metabolic processes which resulted in inhibition of root water channel activity and affected plant water relations. Previous studies have also found reduced water uptake (ergo transpiration) in cattail exposed to 60 mg L^{-1} of NAs after five days of exposure which eventually translated into plant death (Section 3.0). However, because transpiration rates are tightly linked to many other processes within the plant, reduced transpiration is not indicative of what is occurring in the plant but rather is a general indicator of photosynthetic disruption (Trapp et al., 2000). Compounding the efforts to unravel the mechanism of action of NAs in plants is the fact that the acids are a complex mixture of over 200 compounds.

Each individual NA compound within the NA mixture has its own unique physical, chemical, and toxicological properties. Thus the determination of the mechanism of action of these acids in plants is a challenge. Nevertheless understanding the general mechanism of toxic action of this group of chemicals is necessary to help develop phytoremediation systems. For example, plants may be genetically modified to better metabolize or take up toxic contaminants (Arthur et al., 2005; Nandakumar et al., 2005). In some cases the toxic effects may be in part due to indirect toxicity to the root microbial community and may simply require optimization using the appropriate root inoculants (Arthur et al., 2005; Parmar and Dadarwal, 1999).

5.2 Materials and Methods

5.2.1 Chemicals and materials

Unless otherwise noted, all chemicals and materials used in the growth chamber experiment, sample preparation, and analysis were obtained from Fisher Scientific (Edmonton, AB, Canada). Oil sands NA extract was extracted from tailings pond water

using an adapted liquid-liquid extraction method first described by Rogers et al. (2002a). The adapted liquid-liquid extraction method for NAs used here is described in full by Janfanda et al. (2006) and specific details on the extract used in this study is reported previously in Armstrong et al. (2008).

5.2.2 Hydroponic experiments and plant material

Cattail plants were obtained as root cuttings from a native wetland plant nursery (Bearberry Creek Water Gardens, Sundre, AB, Canada) in October 2006. The root cuttings were mass cultured for at least three weeks in plastic containers with ¼ strength modified Hoagland's nutrient medium in an environmental chamber using the growing conditions reported previously (Armstrong et al., 2008). Once the plants were acclimatized to the chamber conditions (~4 weeks), plants that were ~62 cm in length, 7 cm long rhizome, and ~25.7 g in fresh weight were set up in the hydroponic test vessels for one week prior to the start of the experiment. Plants were grown in a hydroponic system originally described by Doucette et al. (2005) and adapted by Armstrong et al. (2008).

For Study 1 plants were grown under control conditions (0 mg L^{-1} NAs) and NAs were spiked into tissue prior to extraction. For FTIR microspectroscopic analysis of root cross sections, plants were grown in either control conditions or exposed to high dose NAs (0 mg L^{-1} and 60 mg L^{-1}, respectively). There were three replicates per treatment (one replicate = one plant in one hydroponic test vessel). Previous work showed greater phytotoxicity when plants were exposed to NAs in their non-ionized form (see Chapter 3.0) therefore to maximize potential uptake plants were grown in medium pH = 5.0. The media was maintained at pH 5.0 and adjusted every five days using 1.0 N HCl or KOH. Plants were harvested after 30 Days.

5.2.3 Plant tissue preparation

To prepare plant tissue (consisting of roots, rhizome, and shoots) for analysis using PLE followed by ESI-MS, the tissue was first dried whole in an oven at 60^{o}C for 12 h. The dried tissue was then cut up in smaller sections using scissors and then

homogenized into a fine powder using a coffee blender. Each plant sample was 0.8 g in dry weight and spiked with 662 µL of a NA spike solution in methanol (MeOH) at a concentration of 0.136 mg mL^{-1}. The spike level chosen was used to represent a maximum concentration expected in plant tissue grown in NA hydroponic experiments of 60 mg L^{-1}. After the MeOH carrier solvent had evaporated the spiked tissue was mixed with 1.6 g of diatomaceous earth (ASE prep-DE, Dionex, Sunnyvale, CA, USA) using a mortar and pestle.

Roots used for FTIR analysis were randomly selected from the plant and sectioned using a stainless steel scalpel from the bottom four centimeters of the root. These sections were immediately immersed in petri dishes containing Tissue Tek OCT (VWR, Edmonton, AB, Canada) and then frozen at -78 ^{o}C. Immediately prior to cryosectioning, ~1 cm section of plant root was removed from within 1 – 3 cm of the plant root tip. This section was isolated for these experiments because it is the area of greatest water uptake in plant roots (Hopkins, 1999). This removed section was then frozen onto specimen blocks using water. The sections were cut using a Lecia CM1900 cryotome set at -20 ^{o}C. The 6 µm thick sections were thaw mounted onto infrared-reflecting "Low-e" glass microscope slides (Tienta Sciences, Indianapolis, IN, USA) and the slides were then stored at room temperature until analysis. Infrared spectra were also collected from the oil sands NA extract and this was achieved by smearing a drop of the extract on an infrared-reflecting slide and letting it dry prior to analysis.

5.2.4 Pressurized liquid extraction and quantification of naphthenic acids

Pressurized liquid extraction was carried out using an ASE 200 (Dionex, Sunnyvale, CA, USA). Samples were prepared in 30 mL stainless steel cells and one glass fibre filter was put into the inlet and one at the outlet of the cell. In between the filters the cell was loosely packed with the plant material/DE mix and then topped up with Ottawa sand. Blanks were prepared using NA – spiked Ottawa sand/DE, instead of plant tissue. Table 1 lists the different PLE parameters that were tried in combination to extract NAs from spiked plant tissue. There are eight different parameters accounted for with PLE used here including: heat time, static time, flush %, purge time, cycles, solvent

choice, pressure, temperature. Of these eight parameters only four (static time, cycles, solvent choice, and temperature) were adjusted here because they were identified as having the greatest influence on sample extraction efficacy (Carabias-Martínez, 2005). There were three plant sample spikes tested for each method and three spiked Ottawa sand blanks tested for each method. Extracts from PLE were collected in 60 mL amber glass vials.

Table 5.1: Settings for pressurized liquid extraction tested for developing a method to extract naphthenic acids (NAs) from plant tissue. Parameters in bold indicate those that were adjusted to optimize extraction of NAs. The pressurized liquid extraction (PLE) Method # 2 (in bold) indicates the method that had the greatest extraction success from a tissue blank (sand).

PLE Parameter	PLE Method # 1	**2**	3	4	5	6	7	8	9	10
Heat	5 min	**5 min**	5 min	5 min	5 min	5 min	5 min	5 min	5 min	5 min
Static Time	5 min	**5 min**	5 min	5 min	5 min	5 min	5 min	5 min	**10 min**	**15 min**
Flush %	30 %	**30 %**	30 %	30 %	30 %	30 %	30 %	30 %	30 %	30 %
Purge Time	120 s	**120 s**	120 s	120 s	120 s	120 s	120 s	120 s	120 s	120 s
# Cycles	2	**2**	2	2	2	2	**1**	**5**	2	2
Solvent	**ACN**	**Basic**	**H_2O**	**MeOH**	Basic	Basic	Basic	Basic	Basic	Basic
Choice		**H_2O**			H_2O	H_2O	H_2O	H_2O	H_2O	H_2O
Pressure	1500 psi	**1500 psi**	1500 psi	1500 psi	1500 psi	1500 psi	1500 psi	1500 psi	1500 psi	1500 psi
Temp.	100°C	**100°C**	100°C	100°C	**50°C**	**150°C**	100°C	100°C	100°C	100°C

ACN – 100% Acetonitrile
Basic H_2O – 0.1% KOH in water
Water – MilliQ water
MeOH – 100% Methanol

Extracts prepared with ACN and MeOH produced a precipitate in the extract which was separate by centrifuging the samples and pouring off the supernatant into a new vial and evaporated to dryness under a N_2 stream. The dried samples were reconstituted in 1.5 mL of 50:50 ACN:H_2O + 0.1% NH_4OH, sonicated for 30 seconds and then transferred to a glass LC vial for analysis. Water PLE extracts were cleaned up using solid phase extraction method described previously (Armstrong et al., 2008). Naphthenic acids in

PLE extracts produced were analyzed using a Quattro Ultima (Waters Corp. Milford, MA, USA) triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface operating in the negative ion mode. Instrument operating parameters are reported previously by McMartin et al. (2004).

5.2.5 Synchrotron fourier transform infrared microspectroscopy

Synchrotron FTIR microspectroscopy was conducted at the Canadian Light Source (CLS) at the University of Saskatchewan in Saskatoon, Saskatchewan, Canada on the Mid-IR 01B1-1 beamline. The beamline has energy levels of 1 x 10^{14} at 10μm (ν s^{-1} /0.1% BW @ 100 mA). The spectroscopic images were recorded using a Bruker IFS 66v/S FTIR spectrometer equipped with a Hyperion confocal microscope and mapping stage controller, a 32x objective, and a DLATGS detector with KBr window (Bruker Optics Ltd., Milton, ON, Canada). The range of the detector was 5000 – 750 cm^{-1} with a resolution of 4 cm^{-1} and an aperture setting of set at ~10 μm x 10 μm. The mapping steps were of the same size as the aperture (~10 μm). The size of the maps taken from plant roots were on average a grid of 10 x 80 individual spectra covering a total area of 100 x 800 μm going from the outside of the root (epidermis) to the centre of the root (stele) (Figures 4 and 5). For the NA standard and background the spectra were produced from the average of 250 scans. For the spectral maps of plant roots, 32 scans were averaged to increase the signal to noise ratio. Each spectrum was ratioed to a background reading taken on an empty part of the slide.

Spectral images were collected from 6 exposed roots and 6 control roots and displayed in absorbance mode and analyzed using OPUS 6.0 software (Bruker, Optics Ltd., Milton, ON). A baseline correction was applied to the final spectra and the spectra were also corrected for water vapour and CO_2. The final absorbance for spectra was expressed as log reflectance (Log [R^{-1}]). Chemical imaging was conducted using the assignment of the IR bands outlined in Table 5.2

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5.3 Results

5.3.1 Pressurized liquid extraction with electrospray ionization mass spectrometry

There were no NAs detected in the spiked plant tissue extracts using PLE followed by low resolution ESI-MS (Figure 5.1). Even when the spectra of blank plant tissue was subtracted from the spectra of plant + NA extract (Figure 5.2a and 5.2b), there was no evidence of the typical NA spectra (Figure 5.1a). Using Method # 2 (Table 5.1; 5 min static time, basic H_2O solvent, 2 cycles, 100 ^oC oven temperature) NAs were successfully extracted from the matrix blank of Ottawa sand spiked with NAs. The mean recovery of the three matrix blank samples was 41.7% ± 21.5% SD.

5.3.2 Synchrotron fourier transform infrared microspectroscopy

The infrared spectra of the NA standard is depicted in Figure 5.3. FTIR microspectroscopy has previously found that NAs extracted from OSPW have two characteristic absorbencies at 1743 cm^{-1} and 1706 cm^{-1} (CEATAG et al., 1998; Holowenko et al., 2002; Yen et al., 2004). In addition to these reported absorptions, carboxylic acids usually have a strong absorbance between 1760 – 1690 cm^{-1} for the C=O stretch as well as a medium absorbance between 3300 – 2500 cm^{-1} for the O-H stretch, 1300-1000 for C-O stretching, and medium absorbance between 950 – 910 cm^{-1} for the C-O-H bend (Stuart, 1997). As far as the absorption bands common to plant tissue, plants are 80% dry weight carbohydrates which is predominantly cell wall (a mixture of cellulose, starches, pectins and sugars). The major mid IR bands of cellulose occur at the following wave numbers: 1170-1150 cm^{-1}, 1050 cm^{-1}, and 1030 cm^{-1} (Stuart, 2004). Cattail is a commeneloid monocot, and therefore has a Type II cell wall. The tissue is a mixture of cellulose and glucuronarabinoxylan polymers, is pectin poor and contains little protein but does contain an extensive network of phenylpropanoid compounds (lignin) between cellulose fibres for added structure (Mitich, 2000; Carpita and McCann, 2000).

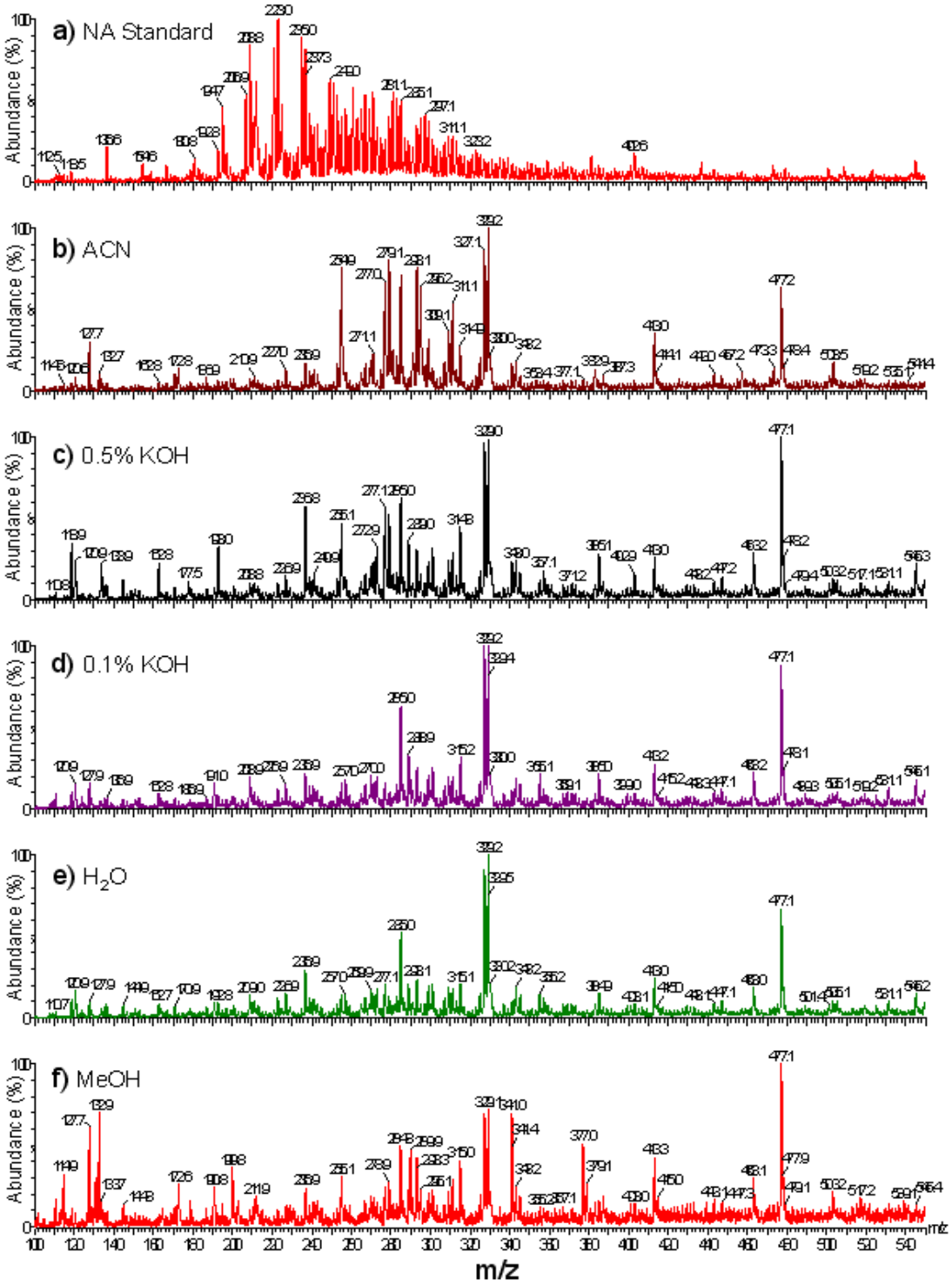

Figure 5.1: Mass spectra for a) oil sands naphthenic acid (NA) extract compared to pressurized liquid extraction extracts of NA-spiked dried cattail tissue using b) acetonitrile (ACN), c) 5% potassium hydroxide (KOH) in water, d) 0.1% KOH in water, e) water, f) Methanol (MeOH) as extracting solvents.

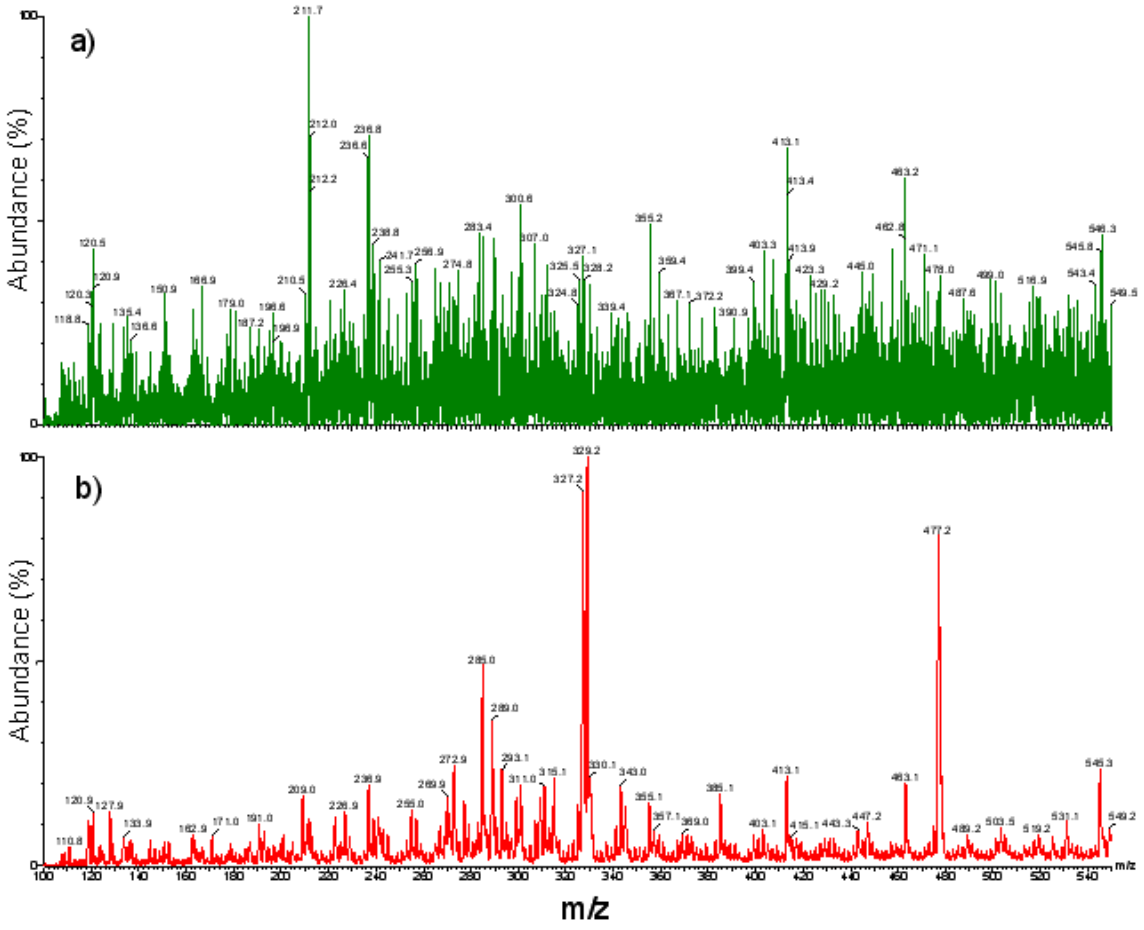

Figure 5.2: a) Difference spectra created by subtracting b) the mass spectra an extract from a control plant tissue from the mass spectra of a naphthenic acid spiked plant tissue extract (Figure 5.1d) both produced using pressurized liquid extraction with a 0.1% potassium hydroxide solvent and the instrument settings listed in Table 5.1, Method # 2.

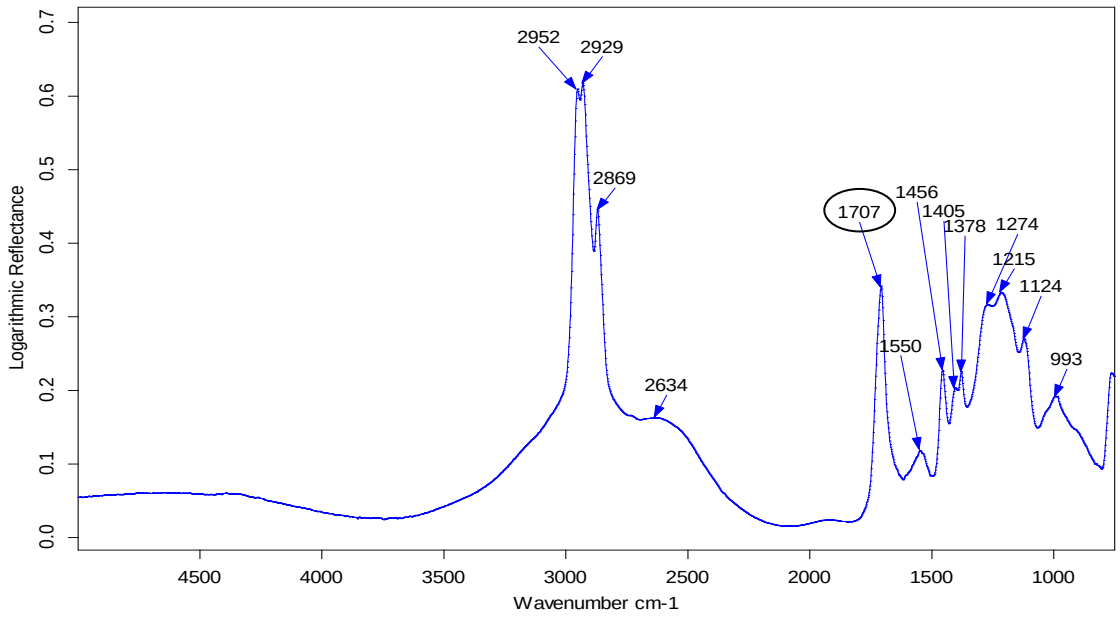

Figure 5.3: Infrared spectra for oil sands naphthenic acids (NAs) extract (pH = 5.0) in 0.1 hydrochloric acid and water solution. Encircled peak at 1707 cm^{-1} wave number is likely that for the carboxylic acid (dimer form) carbonyl (C=O) stretch and the most unique peak within the spectra for potential use as a marker for NAs in plant tissue.

Lignin is anticipated to have absorption bands due to the C-C stretch of aromatic rings at 1590 cm^{-1} and 1510 cm^{-1} (Stuart, 2004). Lipids are dominated by CH_2 stretching and lipid phase transitions may be observed through the absorption band between 2924-2854 cm^{-1}. Proteins are observed through two large Amide bands, Amide I at 1650 cm^{-1} and Amide II at 1550 cm^{-1} (Dokken and Davis, 2007). Callose is a different form of cellulose (chains of [1-3]β-D-glucan rather than the [1-4]β-D-glucan polymers found in cellulose). Callose is produced at specific times in the plant, such as the formation of pollen tubes, cell plates of dividing cells, as well as in the event of wounding (both mechanical wounding and chemical wounding or toxicosis) (Carpita and McMann, 2000). Chen et al. (2007) attributed the absorbance between 980-800 cm^{-1} wavenumbers to callose. In the present study, the absorbance within the range of 980 to 800 cm^{-1} was monitored to determine if callose was produced as a response to NA exposure. Table 2 lists all the peaks which were analyzed in root cross sections.

Table 5.2: Assignment of main infrared bands found in plant tissue and oil sands naphthenic acids extract. Infrared bands indicated with an asterisk identify those frequency ranges that showed the greatest differences between control and exposed roots.

Wavenumber (cm^{-1})	Assignment	Comments	Reference
2952	Asymmetric CH_3 stretch	NA Standard Plant lipids	NA standard (Fig 5.3) Dokken and Davis (2007)
2929	Asymmetric CH_2 stretch	NA Standard Plant lipids (suberin in the endodermis)	NA standard (Fig 5.3) Zeier and Schreiber (1999) Dokken and Davis (2007)
2869	Symmetric CH_3 stretch	NA Standard Plant lipids	NA standard (Fig 5.3) Dokken and Davis (2007)
1760-1690	Carbonyl stretch (C=O)	Carboxylic Acids general (plant tissue)	Stuart (2004)
1707*	Carbonyl stretch (C=O)	NA Standard – C=O	NA standard (Fig. 5.3)
1680-1660	C=O stretch plus C-N stretch	Amide I (Protein; plant tissue)	Dokken and Davis (2007)
1590*	Aromatic in-ring C-C stretch	Lignin (cell wall plant tissue)	Stuart (2004) Dokken and Davis (2007)
1560-1530	N-H deformation plus C-N stretch	Amide II (Protein; plant tissue)	Dokken and Davis (2007)
1510	Aromatic in-ring C=C stretch	Lignin (cell wall plant tissue)	Stuart (2004) Dokken and Davis (2007)
1354-1000	C-O stretch	NA Standard (C-O Stretch)	NA standard (Fig 5.3)
1170-1150*	C-H stretch	Cellulose (carbohydrates – cell wall)	Stuart (2004)
980-800		Callose – cell wall material produced as a wound response	Chen et al. (2007)

Figure 5.4 shows micrographs of a cattail root cross sections with identification of root anatomy to aid in the association of IR maps to specific root components. The most striking feature of the cattail plant root is the large air spaces in the root cortex with spoke-like connections of cortex cells connecting the epidermis to the stele. These air spaces, known as aerenchyma, are an adaptation to the hypoxic aquatic environment of

the plants (Seago Jr. et al., 2005). The aerenchyma permit the diffusion of oxygen from the upper parts of the plant down to the plant roots for cellular respiration. In addition, transferred oxygen protects the plant from absorbing phytotoxic reduced substrates such as Fe^{2+}, Mn^{2+}, and sulphide, by diffusing into the rhizosphere and providing an oxidative layer on the root surface (Matsui and Tsuchiya, 2006).

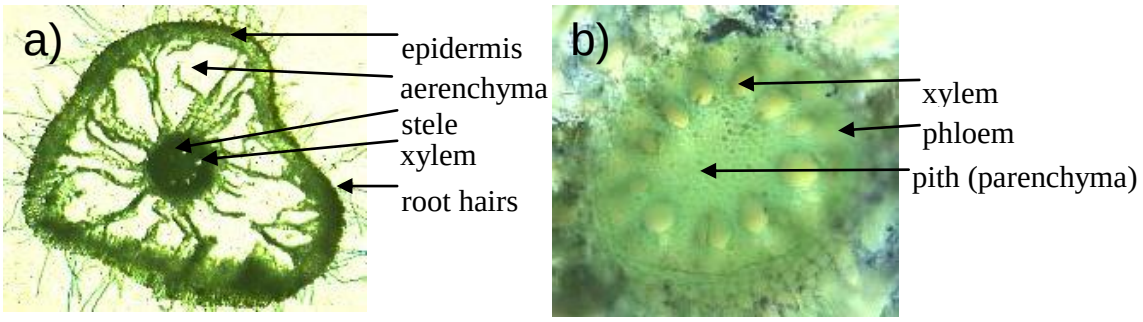

Figure 5.4: a) cross section of a cattail root. b) enlarged image of the central vasculature in the plant root, the stele. Images are courtesy of Dr. John Cheeseman, Department of Plant Biology, University of Illinois and used with permission.

Figure 5.5 depicts the visual images (5.5a and 5.5c) of the representative root cross sections analyzed by synchrotron FTIR microspectroscopy. The red boxes in Figures 5a and 5c indicate the areas of the IR images (Figures 5b and 5d). The IR images displayed in Figures 5.6 and 5.7 are also taken from this same area but represent the signature peak for lignin (1590 cm^{-1}) and cellulose region (1170-1150 cm^{-1}) respectively. The IR peak being investigated in Figure 5 is that which was identified as being a unique identifying peak in the NA standard 1707 cm^{-1} (Figure 5.2, Table 5.2). There are some very striking differences in the IR maps at this wavenumber cm^{-1} between the control (Figure 5.5b) and exposed (Figure 5.5d) root. First of all there is a distinct (~50-70 μm, X-axis) band, of moderate absorbance (0.3), in the outer epidermis of the control root whereas there is a less distinct and a lower intensity (0.2) absorbance in the NA exposed root over a broader range (~50-300 μm) in the epidermal region. For the stele region, defined circles can be seen with zero absorbance in the center. These circles are the xylem (water conducting) elements in both roots. The control has moderate to high

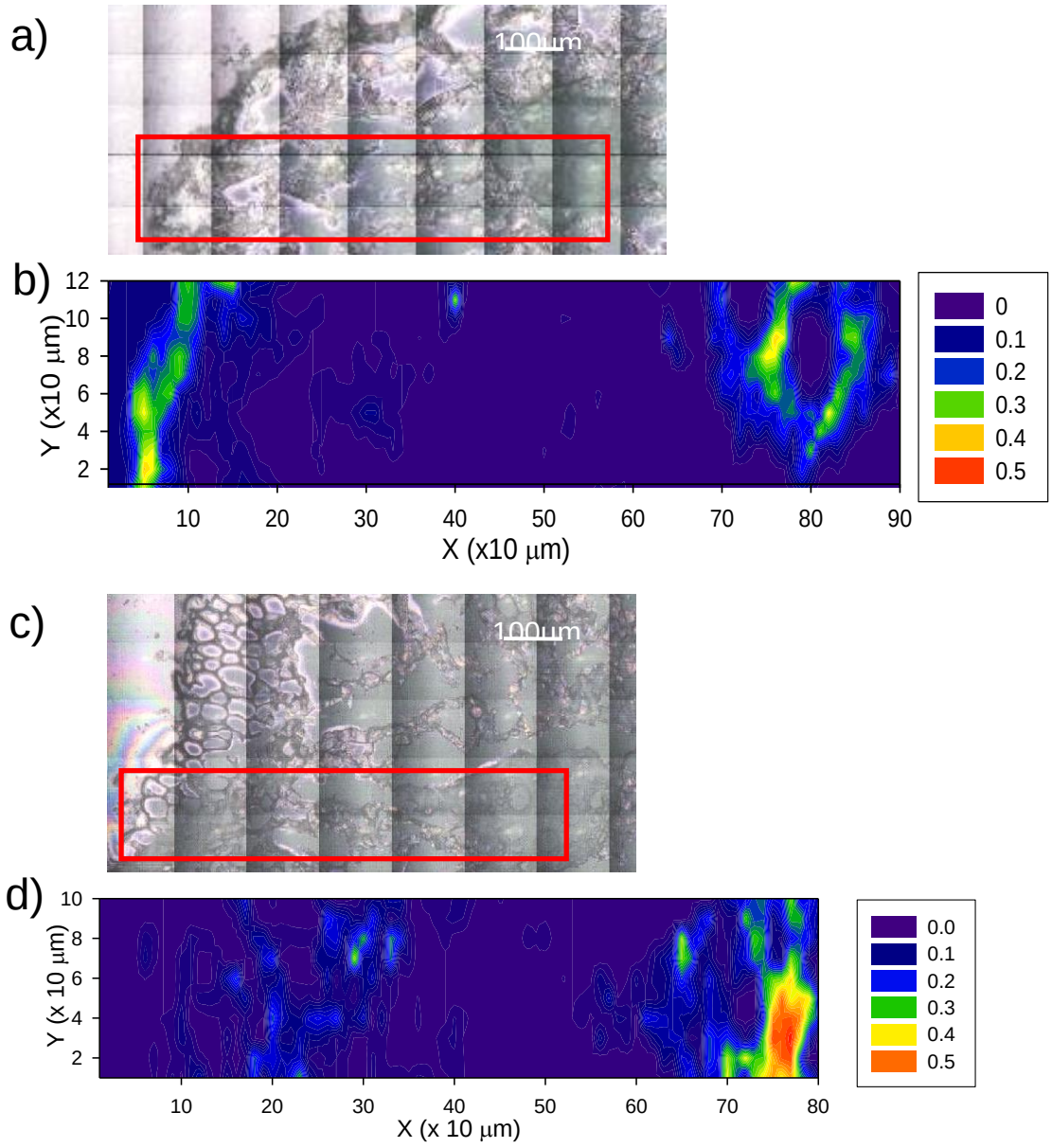

Figure 5.5: Visual images and infrared maps of cattail root cross sections of control roots (a and b) and in roots exposed to oil sands naphthenic acids (60 mg L^{-1}) (c and d). The red rectangle of the visual images (a and c) represents the area mapped with fourier transform infrared (FTIR) microspectroscopy. The infrared maps presented here are of the peak at 1707 cm^{-1} which is found in the naphthenic acid standard. The epidermis is located to the left of the images (between 50 – 250 µm on the X-axis) and then moves towards the stele at the right side of the image (~700-900 µm along the X-axis).

absorbance (0.3-0.4) in the phloem tissue surrounding the xylem. In the exposed root there is intense absorbance (0.4-0.5) in the central pith of the stele as well as some increased absorbance (0.3) in the phloem. In both IR maps there is little or no absorbance in the cortex region of ~300 to 600 μm (X-axis) between the epidermis and stele.

In Figure 5.6, depicting the absorbance of the peak at 1590 cm^{-1} which is typical of the plant biomolecule lignin, there is minimal presence of this peak throughout the control root (Figure 5.6a). There is some low absorbance (0.2) delineating the stele region in the control root. In the NA exposed root (Figure 5.6b) there are more intense peaks at this wavenumber (0.6 – 0.8) with more pronounced delineation of the stele between 600 – 800 μm along the X-axis. There is also some absorbance of this peak into the central root cortex region between 200-600 μm (Figure 5.6b). In general, of the two single peaks investigated here in Figures 5.5 and 5.6, the peak at 1590 cm^{-1} has the highest absorbance reaching an intensity of up to 0.8 (Figure 5.6b).

Figure 5.7 depicts the presence of cellulose which absorbs in the region of 1170-1150 cm^{-1}. Because cellulose absorbs within the complex finger print region (~1500-600 cm^{-1}; Stuart, 1997) it is more accurate to integrate the area under the peak within this range (1170-1150 cm^{-1}) rather than just report its log reflectance intensity as was done in Figures 5.5 and 5.6. Over the range of cellulose increased presence is found primarily in the area of the stele in both control and exposed roots (Figures 5.7a and 5.7b, respectively). As in Figure 5.5d for the 1707 cm^{-1} absorbance, there is an increased absorbance in the central pith of the stele in the exposed root (~750 μm, X-axis, Figure 5.7b).

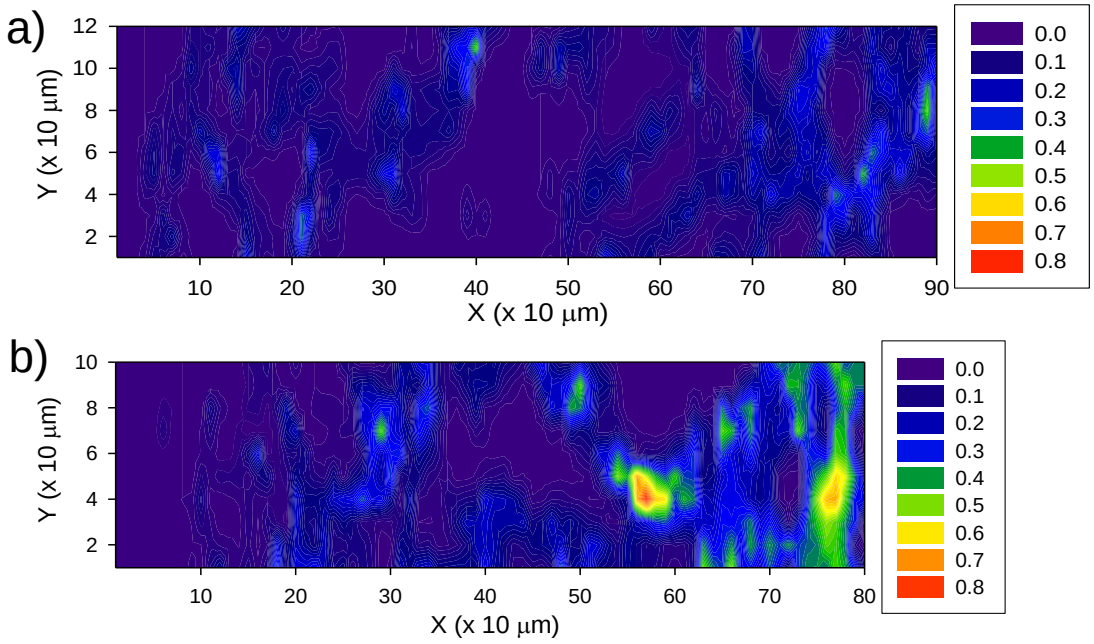

Figure 5.6: a) Infrared maps of cattail root cross sections of a control root and b) a root exposed to oil sands naphthenic acids (60 mg L^{-1}). These infrared maps are taken from area outlined in red in Figure 5.5a and 5.5c. The Infrared maps presented here are of the peak at 1590 cm^{-1} which is typical of the plant biomolecule lignin. The epidermis is located to the left of the images (between 50 – 250 μm on the X-axis) and then moves towards the stele at the right side of the image (~700-900 μm along the X-axis).

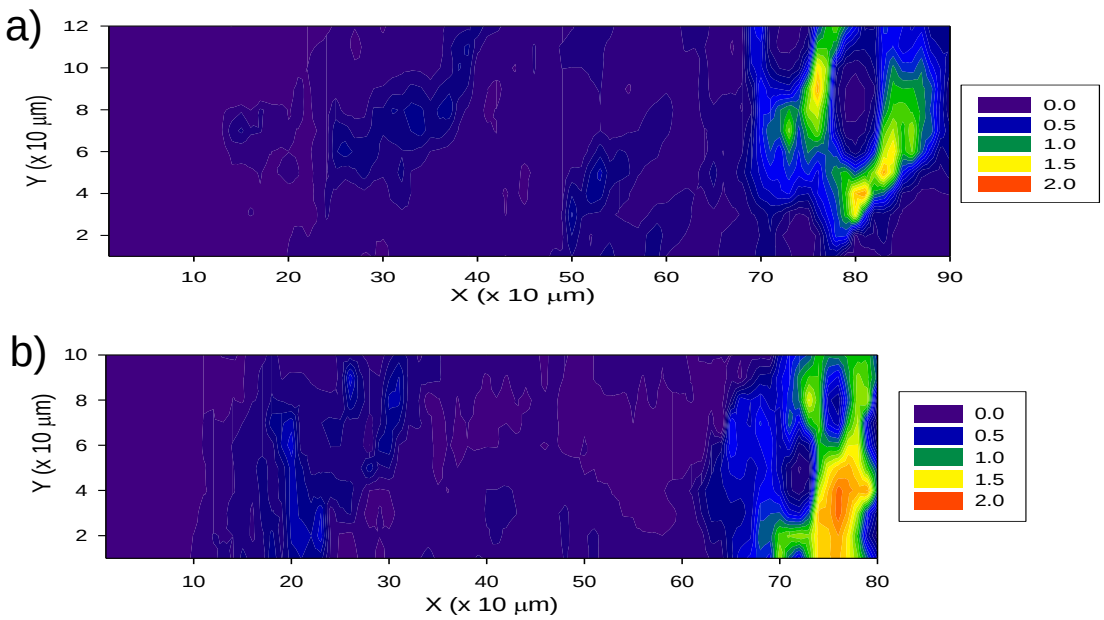

Figure 5.7: a) Infrared maps of cattail root cross sections of a control root and b) a root exposed to oil sands naphthenic acids (60 mg L^{-1}). These infrared maps are taken from area outlined in red in Figures 5.5a and 5.5c. The infrared maps presented here are of the integrated area under the peak between the wavenumbers 1170-1150 cm^{-1} which is typical of the plant biomolecule cellulose. The epidermis is located to the left of the images (between 50 – 250 µm on the X-axis) and then moves towards the stele at the right side of the image (~700-900 µm along the X-axis).

5.4 Discussion

5.4.1 Pressurized liquid extraction with electrospray ionization mass spectrometry

The different PLE methods used here appeared to be unsuccessful at separating NAs from plant tissue. It may be that NAs are masked by co-eluting plant compounds that also have weak acid functional groups (the chemical group in NAs that was capitalized on here for extraction). This could include compounds such as: fatty acids, amino acids, malonic acid, etc. However even when the spectra of a blank plant tissue extract is subtracted from the spectra of a NAs plus plant tissue extract (Figure 5.2), no evidence of the typical NAs molecular fingerprint (Figure 5.1a) appears. In the case of

the extracts where the solvents MeOH and ACN were used, a precipitate was formed in the bottom of the extract which was separated by centrifugation and pouring off the supernatant in an LC vial for final analysis by ESI-MS. This precipitate is likely composed of plant proteins as these solvents are commonly used to precipitate proteins. In this situation the NAs could be bound to the precipitate that formed in these extracts and would not therefore be recovered in the final supernatant which was analyzed by ESI-MS. Young et al. (2007) developed a method for the analysis of NAs in fish tissue. The authors acidified the homogenated tissue and then used a liquid-liquid extraction method with a 2:1 mixture of the solvent chloroform:methanol. This solvent mixture was not tested here and should be considered for future study. Also for future study: high resolution or ultrahigh resolution mass spectrometry of the extracts produced using PLE, which may actually reveal the detection of the NA components in the given extracts. Likewise some spatial separation using HPLC with ESI/MS could also be successful here.

5.4.2 Synchrotron fourier transform infrared microspectroscopy

The 1707 cm^{-1} wavenumber identified here as being the carbonyl stretch in the carboxylic acid functional group of the oil sands NA extract is slightly different than the 1706 cm^{-1} reported in the literature previously for a commercial extract of these compounds (Yen et al., 2004). Also at 1743 cm^{-1} wavenumber, previously identified to be the monomer form of the carboxylic acid (CEATAG, 1998; Holowenko et al., 2002), is absent in the spectra of the extract used in the present study. Both of these observations are likely a result of several factors including: exact source of the NAs (commercial extract vs. oil sands NA extract), and the solvents used to prepare the extract (water at pH = 5.0 used here vs. methylene chloride).

The IR maps in control and exposed roots for 1707 cm^{-1} wavenumber demonstrated some evidence for both physiological effects of NAs and potential uptake and absorption into plant tissue. The first notable difference between the control roots is present in the epidermis region (50-250 μm) absorbance being displayed here is likely a result of co-absorption of organic acid root exudates being produced in the epidermal cells of the control root (Dakora and Phillips, 2002). The carbonyl C=O stretch of

carboxylic acid compounds absorbs in general between 1760-1690 cm^{-1} wave numbers (Table 5.2). Therefore it is very likely that an endogenous organic acid production is occurring here. What is most interesting about this observation; however, is its absence in the exposed root. The absence of absorbance most likely indicates compromised root function and most likely cellular toxicity to the root epidermis as a result of NA exposure. This could be of great significance to plant health because the root epidermal cells are critical for uptake (and regulation) of not only water but of plant nutrients as well as protection from the surrounding environment (chemicals, pathogens, etc.) (Hose et al., 2001).

In the stele of the control plant root in Figure 5.5b the increased absorbance at the 1707 cm^{-1} wavenumber is likely again the result of endogenous carboxylic acid compounds (metabolites) being transported in the phloem. The phloem is used by the plants to transport photosynthetic products and metabolites to areas of active metabolism in growing roots (Hopkins, 1999). In the exposed root (Figure 5.5d) there is greater IR absorbance at 1707 cm^{-1} in the phloem tissue surrounding the xylem elements than the control root. Furthermore, there is intense absorbance observed within the pith area of the root. The pith consist of undifferentiated, meristematic, parenchyma cells which have a large central vacuole and mainly serve for storage and wound healing in the root (Hopkins, 1999). Therefore two possible hypotheses may account for the observations reported here. The increased absorption in the pith could be evidence that the NAs are (a) being taken up and stored in the cells and (b) transported by the phloem to other parts of the plant. Another possible explanation for this absorption is that because of the compromised function of the roots epidermis as a result of NA exposure, the plant may need to mobilize any nutrient stores in the parenchyma cells in the pith to help support its growth. It is also likely that both hypothesis are occurring simultaneously. However if the nutrient mobilization was taking place one would expect to see a decrease in intensity of peaks at wavenumbers indicative of energy-storing compounds such as plant lipids (e.g. 2952 cm^{-1}, 2929 cm^{-1}, and 2869 cm^{-1}; Table 5.2) which does not appear to occur (data not shown).

Mapping the absorbance of the wavenumber indicative of lignin (1590 cm^{-1}) suggest that there is scattered presence of this molecule throughout the control root.

Such a distribution would be expected because of the cross-linking structural function between cellulose polymers in cell walls (Carpita and McMann, 2000). In the stele region of the plant root there is a slight increase in absorbance here which would be expected since this area is highly structurized to provide the tensile strength needed for the water conducting elements in the xylem. In the exposed root; however, the pattern for lignin in the IR map at 1590 cm^{-1} changes. There is increase absorbance not only around the stele but also through the aerenchymous cortex. This distribution could be a combination of several factors. 1) The increased intensity could be an indirect effect of the NA toxicity to root cells. The decreased presence of competing biological molecules in living cells improves the absorbance of the more resilient lignin biomolecule remaining in cellulose. 2) Naphthenic acids are being metabolized and incorporated into the plant tissue as lignin structure which has been suggested to occur previously in benzotriazole exposed sunflower (*Helianthus annus*) roots (Dokken et al., 2005). 3) The plant is producing aromatic secondary plant metabolites as a defense response to the NA exposure. Plants have a systemic response to chemical toxicity that is similar to mechanical wounding by herbivory or bacterial infection (Hammond-Kosack and Jones, 2000). Many secondary plant metabolites are aromatic compounds (Croteau et al., 2000) and since the 1590 cm^{-1} wavenumber used here to identify lignin is actually of the aromatic ring C-C stretch (Table 5.2) the later may be contributing to absorbance seen at this wavenumber. 4) Finally the increased absorbance in this region could be evidence of NA uptake and presence in the plant tissue. Although the structural definition of NAs does not include the presence of unsaturated ring structure (such as aromatic compounds), aromatic groups may be present as impurities in oil sands derived NAs (Dorn et al., 1992; Mojelsky et al., 1992; CEATAG, 1998). The presence of aromatic NA like compounds could thus contribute to the absorbance of the 1590 cm^{-1} lignin peak. In the spectra of the NA extract (Figure 5.1) there is a broad peak at 1550 cm^{-1} which may also contribute to the absorbance at 1590 cm^{-1}. Of the four plausible options presented for this wavenumber; 1590 cm^{-1}, the second and third scenarios (i.e. NAs are being metabolized and incorporated into the plant tissue as lignin structure and that the plant is producing secondary plant metabolites as a defense mechanism) is likely the most accurate. Armstrong et al. (1996) also reported increased lignification of the

emergent macrophyte common reed (*Phragmites australis*) roots upon exposure to organic acids. Increased lignification in response to NA exposure ultimately may result in blockages in the water conducting elements in the plant ultimately leading to water and mineral stress in the plant (Armstrong et al., 1996).

The final wavenumber that showed the greatest differences between control and exposed roots that was investigated from Table 5.2 was that for cellulose (1170 – 1150 cm^{-1}). The presence of cellulose is expected throughout the plant root because of its dominance in cell wall material. However, the area for which this compound absorbs coincides with the fingerprint region of the IR spectrum (1500-600 cm^{-1}) where many other peaks from other organic compounds overlap (Stuart, 1997). Therefore only the most cellulose-rich area dominates these IR maps, which is the stele. The images of the stele in these IR maps is similar to that seen in Figure 5.5, in that the NA exposed cell has high absorption in the area of the central pith containing parenchyma cells. There may therefore be absorbance occurring as a result of the either absorbed NA compounds or other endogenous organic acid compounds due to the C-O stretch which occurs between 1300 and 1000 cm^{-1} (Stuart, 1997).

The analysis of plant roots exposed to oil sands NAs by synchrotron FTIR microspectroscopy has potential for exciting advancement. Quantitation was outside the scope of this preliminary investigation; however, more research needs to be conducted with dose response exposures combined with comparisons to calibration curves created with NA standards of known concentration to determine if quantification is possible in plant tissue. In order to better elucidate effects of NAs in plant tissue, future work should also compare and contrast the IR maps of exposed roots at 5 days post exposure vs. those at 30 days post exposure investigated here. Finally, studies with protoplasts (plant cells in suspension minus their cell wall) may help determine the exact fate and mechanisms of action that NAs have on plant cells.

5.5 Conclusions

Preliminary results were obtained for extraction of NA from plant tissue using PLE followed with analysis by ESI-MS. Work is warranted to continue method development with different solvents used for the extraction of NAs from fish tissue. Also high resolution mass spectrometry analysis methods currently under development may also provide a lower detection limit and improved ability to discern NAs from competing plant compounds co-extracted during PLE.

The findings observed using the synchrotron FTIR microspectroscopic analysis plant roots exposed to NAs are also of a preliminary nature. There were observable differences at the 1707 cm^{-1} wavenumber which was identified as a signature absorbance for this group of compounds (despite the differences amongst the individual compounds within the mixture). If the 1707 cm^{-1} wavenumber, is due to NA uptake and presence in plant tissue, then it appears that NAs absorb in the areas of the plant root that one expects weak acids to accumulate for transport or storage (phloem, parenchyma). Increase in the 1590 cm^{-1} wavenumber absorption indicates that the plant roots undergo increased lignification in response to NA exposure ultimately increased lignification may result in blockages in the water conducting elements in the plant ultimately leading to water and mineral stress in the plant. This is the first report of NAs causing increased lignification in plants.

With regards to the effects of NAs on root tissue as observed in by synchrotron FTIR microspectroscopy, this technique was able to detect some changes in the chemistry of the cattail roots. Specifically, changes were observed in the outer cell layer of the plant roots, the epidermis. Secondly, changes were noted that could be attributed to structural changes in lignin as well as changes in the parenchyma cells located within the root pith. Further investigation is needed to determine what these changes mean as far as NA mechanisms of action and overall plant health.

6.0 Phytotoxicity and Naphthenic Acid Dissipation from Different Oil Sands Process Water Treatments Planted with the Emergent Macrophyte *Phragmites australis*

6.1 Introduction

The extraction of oil from bitumen is heavily reliant on water, requiring approximately 12 m^3 of water for every 1 m^3 of crude oil produced. After water recycling there is a net production of approximately 4 m^3 of slurry waste (Mikula et al., 2008). As of 2002, approximately 120 million barrels (19 million m^3) of sweet crude oil was produced annually from the Athabasca oil sands industry. This annual production is expected to rise to 400 million barrels (64 million m^3) per year as traditional sources of petroleum deplete and greater demand is placed on other oil sources such the oil sands (Holowenko et al., 2002). The combination of increasing demand for oil and a zero discharge policy for oil sands process water (OSPW) has increased the urgency to develop methods to remediate OSPW to reduce impacts on local hydrology (Chalaturnyk et al., 2002; Elshorbagy et al., 2005; Schindler and Donahue, 2006).

Oil sands processed water is actually a clay suspension made up of 70% water (by weight) and 30% weight solids with greater than 90% of these solids less than 44 μm in size (Mikula et al., 1996). As a result of this property, OSPW clay suspensions consolidate very slowly (estimated to be thousands of years for full consolidation (Mikula et al., 1996)). Water chemistry can be manipulated to produce an OSPW suspension that consolidates and dewaters at a much faster rate (e.g. Chalaturnyk et al., 2002). For example, development of several different treatments are on-going at Natural Resources Canada CANMET Energy and Resources Centre in Devon, Alberta to improve the consolidation of OSPW including the addition of various combinations of lime, gypsum, and polyacrilimide polymers.

Constructed wetland treatment systems containing emergent macrophytes have been successful at phytoremediating natural gas storage (Kanagy et al., 2008) and brackish oil field (Murray-Gulde et al., 2003) produced waters. The present study

investigated if a similar strategy could be used to remediate OSPW. In addition to assisting in the dewatering of OSPW through evapotranspiration (Goulden et al., 2007), emergent macrophytes could assist in the removal of toxic organics [e.g. naphthenic acids] through the support of microorganisms in their rhizosphere that promote degradation (Biryukova et al., 2007) as well as through direct uptake and biotransformation in their biomass (Armstrong et al., 2008).

Naphthenic acids (NAs) are a group of alkane and cycloalkane organic acid compounds that are found naturally in oil sands deposits (for review see Headley and McMartin, 2004; Clemente and Fedorak, 2005). During the caustic hot-water extraction of oil from the bitumen in oil sands deposits, NAs become concentrated to 40 – 120 mg L^{-1} in the resulting OSPW (Holowenko et al., 2000). Naphthenic acids are a concern in OSPW because there are acutely toxic to fish and aquatic invertebrates (Headley and McMartin et al., 2004; Clement and Fedorak, 2005) Additionally NAs are water soluble at the alkaline pH conditions of tailings ponds and have the potential to migrate into surrounding aquatic environments (Chalaturnyk et al., 2002.).

Previous studies investigating the uptake of NAs in plants have been conducted exclusively with NAs in hydroponic media to help characterize the dissipation and phytotoxicity of NAs without other interfering toxic components (Armstrong et al., 2008). In actuality, OSPW contains many other potentially phytotoxic components including: salts, metals, and unrecovered hydrocarbons (Mikula et al., 1996). The current study provides insight into how emergent macrophytes would perform in more realistic NA exposure scenarios and assesses what contribution NAs play towards overall OSPW phytotoxicity.

The emergent native macrophyte common reed (*Phragmites australis* subsp. *americanus*) was used as a test species in the present study. This species has two closely related sub species the native *americanus* and the non-native *australis*. The non-native European sub species is more vigorous than the native subsp. *americanus* tive sub species and can become a noxious weed threatening native flora (Hansen et al., 2007). However, common reed is known to grow well in alkaline and brackish conditions; similar to the water conditions observed in OSPW (Mal and Narine, 2004). As well, common reed has an extensive global distribution (Mal and Narine, 2004; Hansen et al., 2007). It is

therefore anticipated that this species will grow in alkaline brackish OSPW in the range of climates within the region of the Alberta's oil sands as well as potential applications to treat OSPW in other countries with oil sands operations.

6.2 Materials and Methods

Hydroponic experiments were carried out using the native emergent macrophyte *Phragmites australis* (common reed). The first hydroponic experiment was carried out with diluted tailings treatments to determine the phytotoxicity and NA concentrations in these treatments over 30 days. A second hydroponic experiment was also conducted using simulated runoff water prepared from, chemically amended fine tailings that had been dried. The purpose of the second study was to determine the leachability of ions from the fine tailings in OSPW once they have become consolidated and dried. It is important to know if these chemical amendments increase the phytotoxicity fine tailings to ensure proper tailings management by oil sands operations.

6.2.1 Chemically amended fine tailings samples and simulated runoff water

All tailings samples and simulated runoff water samples were prepared at CANMET, Natural Resources Canada, in Devon, Alberta. For the first phase of the study the following treatments were used: unamended fine tailings (FT), FT amended with 0.5% lime; FT amended with 0.25% lime and 0.25% gypsum; and FT amended with 0.5% gypsum. For the hydroponic experiments, 5 L of liquid consolidated fine tailings was vigorsly mixed by stirring for 5 minutes with 15 L of tap water. The mixtures were then permitted to settle for one week and then added to the respective hydroponic systems.

For the second phase of the study, three 140 kg samples of fine tailings was either left untreated or chemically amended with either 17.6 g of dry polymer (LT27A, a high molecular weight, medium charge density polyacrilimide) or with 400 g each of lime and

gypsum. The fine tailings treatments (both amended and non-amended) were then spread to a depth of less than 0.5 m and allowed to dry. To simulate runoff water, the dried fine tailings were then sprinkled with 39.72 kg of tap water at a rate of 3.3 kg min^{-1}. The water was left to sit for 25 minutes and then poured off. For the untreated, polymer amended, and 0.25% lime and gypsum amended dried fine tailings 69.7%, 64.1%, and 72.3% of the added water was collected from the dried tailings respectively. The remaining water that was added to the sample was absorbed. Water chemistry for the runoff water was determined using ion chromatorgraphy (IC) MS for ions and inductively coupled plasma (ICP) MS for cations, following standard methods.

6.2.2 Hydroponic experiments

Plants were obtained as root cuttings from a native wetland plant nursery (Bearberry Creek Water Gardens, Sundre, AB, Canada) in October 2006 for the first phase of the study and in July 2007 for the second phase of the study to assess the phytotoxicity of simulated runoff water from fine tailings. The experimental set up, methods, and growing conditions used for the present study are previously published (Armstrong et al., 2008). Root cuttings were mass cultured for at least three weeks prior to the start of each experiment to acclimate to chamber conditions. At the start of the experiments, the plants were a single shoot, ~70 cm long, and were approximately ~15 g in fresh weight. Planted treatments contained plants in the hydroponic testing system; where as unplanted treatments were not planted and were composed only of the testing medium (OSPW treatment) in the hydroponic testing system. Each planted treatment contained three plant replicates (one replicated is equal to one plant in an individual testing system) and three unplanted replicates to determine the influence of plants on NA dissipation. In addition to the chemical amendment treatments, there was a planted treatment in ¼ strength Hoagland's nutrient medium (Armstrong et al., 2008) to use as a phytotoxicity control system. There was also one treatment of hydroponic medium spiked with an oil sands NA extract at a nominal dose of 60 mg L^{-1} at pH 7.8 (actual maximum NA concentration detected in medium = 52.1 mg L^{-1}) to determine the contribution of NAs to phytotoxicity of fine tailings treatments. The oil sands extract used here was extracted from OSPW and exact methods for its preparation are reported

elsewhere (Armstrong et al., 2008). Hydroponic test vessels were randomly located in the growth chamber.

A closed-cell plastic foam plug which fitted snug to the opening of the test vessel was used to support the plants and to keep the evaporation of hydroponic medium to a minimum. Evapotranspiration of the hydroponic medium was monitored (by measuring water uptake (Trapp et al., 2000)) and topped up every 5 days using the same tailings treatment. On Days 0, 5, 10, 20, and 30, a 2 mL hydroponic medium sample was collected for NA analysis. Prior to collection, the hydroponic medium was stirred for two minutes with a magnetic stir bar and the sample was then placed into a 2 mL glass vial. Plant fresh weights were recorded on Days 0 and 30 to determine the fresh weight gain or loss in the plants over the course of the 30 day experiment.

6.2.3 Naphthenic acid analysis

Hydroponic medium samples containing either diluted chemical amendments (Phase 1) or simulated runoff water from dried chemically amended fine tailings (Phase 2) were analyzed for NAs using a Quattro Ultima (Waters Corp. Milford, MA, USA) triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface operating in the negative ion mode. Instrument operating parameters are reported elsewhere (McMartin et al., 2004). Preliminary studies found that hydroponic medium samples could not be directly injected for accurate analysis due to background ion interference (data not shown). As a result, samples were cleaned up prior to analysis, using ENV+ solid phase extraction (SPE) cartridges (Biotage, Chartlottesville, VA, USA) (Headley et al., 2002). Here a 1.5 mL of sample was concentrated to 1 mL using SPE procedures described in a previous study (Armstrong et al., 2008) in a final solution of 50:50 acetonitrile:water and 0.1% NH_4OH.

6.2.4 Data analysis

Data were tested for normality and homogeneity of the variances using 1-Sample Kolomogorov-Smirnov (K-S) test and Levene's tests respectively. Data that was not found to be normal or homogeneous was log-transformed. Growth (dependent variable)

was tested for differences between treatments (factor) using an analysis of variance (ANOVA). Specific differences between treatment means were determined using a Tukey test for post-hoc analysis. On Day 30 differences in water uptake (dependent variable) between control plants and plants in treatments (factor) were also tested using ANOVA. Differences in NA concentration (dependent variable) between treatments (factor) was assed with ANOVA and differences within treatments between planted and unplanted treatments were determined using t-tests. All statistical analyses were carried out using SPSS 13.0 (SPSS Inc., Chicago, IL, 2004). All graphs were created using SigmaPlot 8.1 software (SPSS Inc., Chicago, IL, 2002).

6.3 Results

Regardless of the treatment (either diluted tailings or simulated runoff) all OSPW-affected water significantly reduced plant fresh weight gain and water uptake over 30 days (Figures 6.1 and 6.2). Simulated runoff water treatments appeared to have a greater detriment to plant growth than the diluted fine tailings treatment (Figure 6.1). In the present study there was ~0% fresh weight gain and 0 mL water uptake by Day 30 for three individual treatments (a) fine tailings chemically amended with 0.5% lime, (b) simulated runoff water from unamended fine tailings, and (c) simulated runoff water from fine tailings amended with polymer (Figure 6.1). All fine tailings (whether dilute or simulated runoff water) treatments significantly decreased the water uptake of the plants by Day 30 (Figure 6.2). The NA concentration appears to contribute to the observed phytotoxicity within the tailings treatments, as indicated by the correlation of NA concentration vs. fresh weight gain. As shown in Figure 6.3, there is a significant correlation if an outlier is removed (r^2 = 0.71 vs. 0.51; Figure 6.3). Likewise there is a non-linear negative relationship observed between increasing pH and fresh weight gain (Figure 6.4).

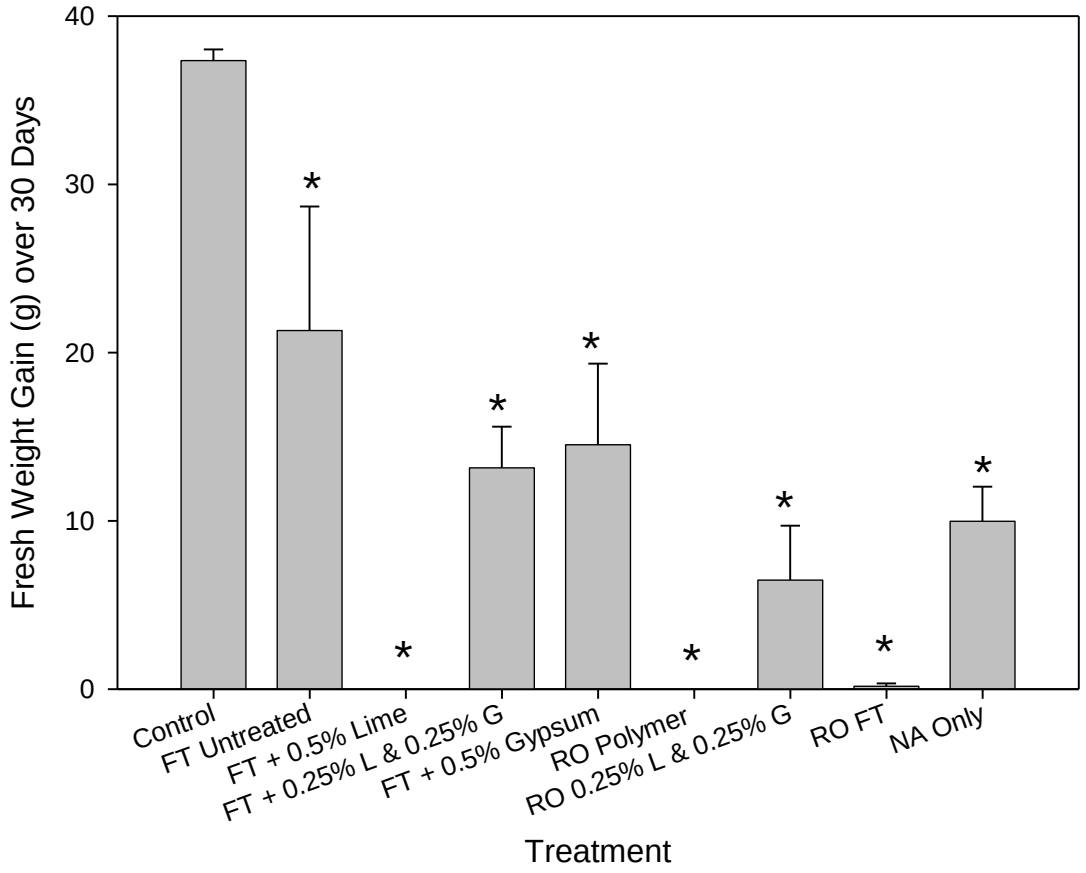

Figure 6.1: Fresh weight gain (g) in common reed over 30 days in different tailings treatments versus a control planted in hydroponic medium. FT = fine tailings. All tailings treatments are made up of FT diluted with 75% water except for simulated runoff water treatments (RO). Simulated runoff treatments are prepared by dripping water on dried FT and collecting the runoff after 25 minutes. Values are reported as the mean ± standard deviation (n = 3). Significant differences from the control group are indicated with an asterisk (*, $P < 0.05$).

As shown in Figure 6.5, there was no significant enhancement of NA dissipation over the course of the 30 day experiment for planted treatments containing diluted tailings or simulated runoff water from tailings. In general there was an increase in NA concentration detected in the planted treatments by Day 30. However, there were no significant differences in NA concentration by Day 30 between planted and unplanted treatments (Figure 6.5). Naphthenic acid concentrations on Day 0 were the highest for

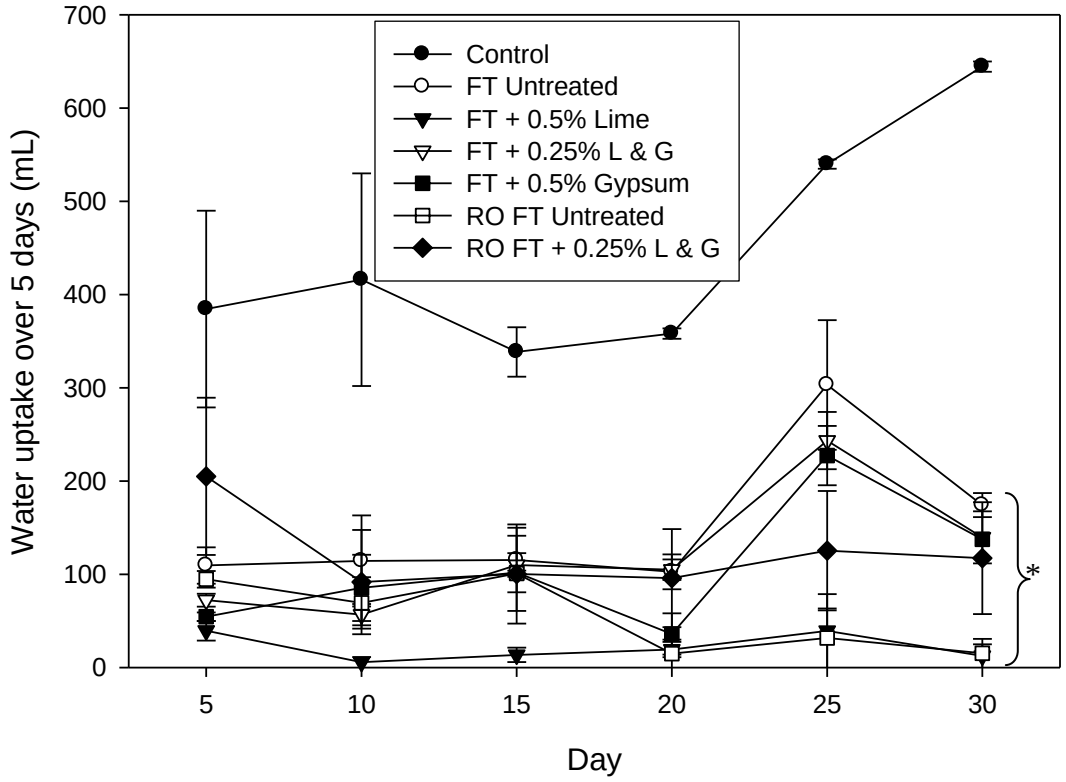

Figure 6.2: Water uptake by common reed as a result of evapotranspiration over five day intervals (mL) grown in either oil sands fine tailings treatments (FT), simulated runoff (RO) from fine tailings treatments, and a control grown in hydroponic medium for 30 days. Values are reported as the mean ± standard error (n = 3). Volume transpired was determined by the amount of hydroponic nutrient solution (mL) required to return the test vessel volume to 2.5 L over five day intervals. The results are reported as the mean (n = 3) ± standard error. Significant differences in water uptake from the control plants on Day 30 are indicated with an asterisk (*; $P < 0.05$). Treatments where the plants died and there was zero water uptake by Day 30 have not been plotted (RO treatments for untreated fine tailings and polymer treated tailings.). L = Lime, G = Gypsum.

treatments with 0.5% lime and the simulated runoff from tailings treated with 0.25% lime and gypsum. In contrast, NA concentration was the lowest for fine tailings treated with 0.5% gypsum (Figure 6.5).

There was a significant effect on the concentration of NAs detected in the tailings treated with either lime or gypsum. The 0.5% lime treatment resulted in a 40 % increase

in NA concentration compared to untreated tailings with a measured value of approximately 60 mg L^{-1} NAs on Day 0 (Figure 6.5). In contrast, much lower concentrations of NAs of approximately 25 mg L^{-1} and 30 mg L^{-1} were observed for chemical amendments with gypsum (including the combination of 0.25% lime and gypsum combined) (Figure 6.5). A similar trend of increased NA concentrations with the chemical amendment of lime was noticed in the simulated runoff water treatments (Figure 6.5). Here the greatest NA concentration of all of the treatments was observed in the simulated runoff water from fine tailings amended with 0.25% lime and 0.25% gypsum.

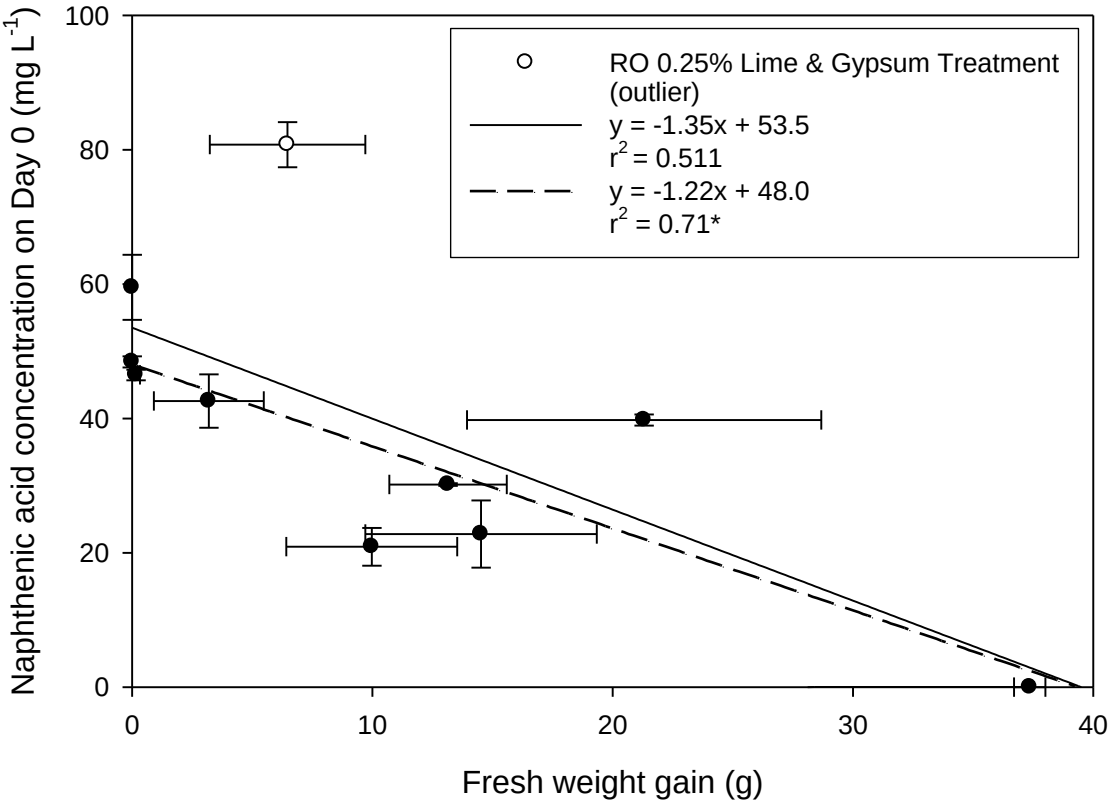

Figure 6.3: Fresh weight gain (g) over 30 days in oil sands tailings treatments planted with Phragmites australis (X) plotted against the starting naphthenic acid concentration (mg L^{-1}) of tailings treatment (Y). Fresh weight and naphthenic acid concentrations are reported as the mean ± SD (n = 3). Two linear functions are plotted on the figure. The first function (—) contains the outlier (O) and the second function (--) is plotted with the removal of the outlier (Runoff (RO) treatment of 0.25% lime and gypsum). Significant r^2 value is indicated with an asterisk (* ; $P < 0.05$).

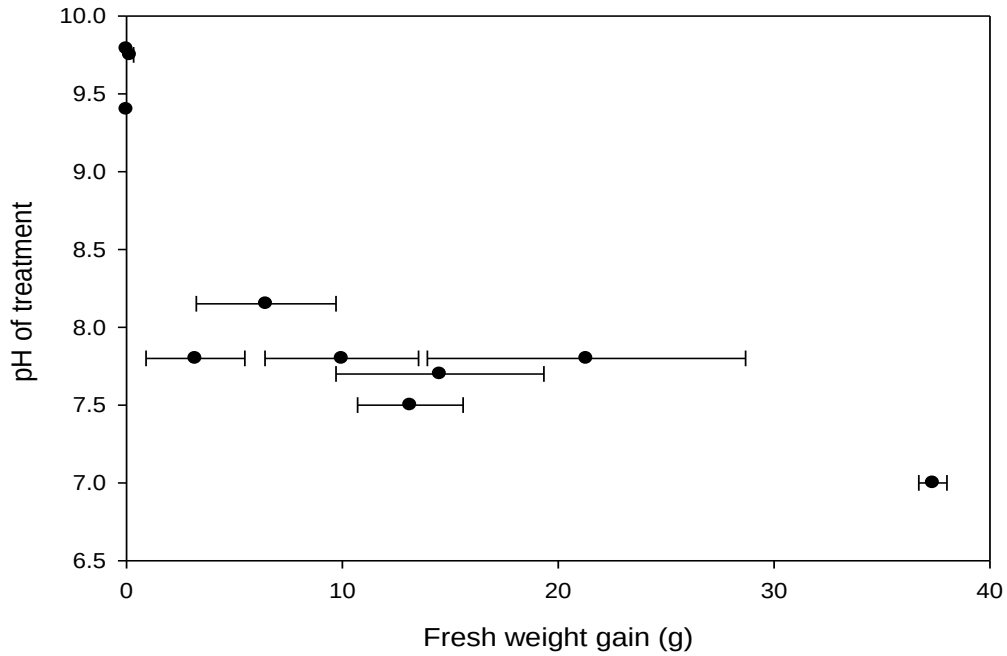

Figure 6.4: Fresh weight gain (g) over 30 days in oil sands tailings treatments planted with common reed (X) plotted against the starting pH of treatment (Y). Fresh weight is reported as the mean ± SD (n = 3)

Table 6.1: Final water chemistry variables for the various tailings and runoff treatments compared to the pore water in the original fine tailings and tap water used to prepare the runoff.

Sample ID	Ca^{+}	K^{+}	Mg^{+}	Na^{+}	S^{2-}	Fe	Cl^{-}	SO_4^{2-}	HCO_3^{-}	CO_3^{2-}	OH^{-}	pH	IB
Untreated MFT diluted	4	9	1	51	8	0	13	29	100	0	0	7.9	1.05
0.5% Lime MFT diluted	19	6	2	39	17	2	8	50	89	0	0	7.8	1.09
0.25% Lime and 0.25% Gypsum MFT diluted	54	6	2	66	86	0	10	247	46	0	0	7.8	0.96
0.5% MFT Gypsum diluted	59	6	2	27	62	0	5	176	43	0	0	7.0	0.99
Polymer MFT Runoff	14	11	21	517	87	0	337	220	302	146	0	9.79	1.05
Untreated MFT Runoff	12	10	16	464	57	0	331	130	318	139	0	9.75	1.03
0.25% Gypsum and Lime MFT Runoff	372	17	27	1591	1037	0	764	2903	246	0	0	8.15	1.05
Initial MFT (Pore Water)	17	16	8	744	16	0	333	22	1535	8	0	8.33	0.97
Tap Water	47	1	16	12		0	0	80	149	0	0	7.78	1.03

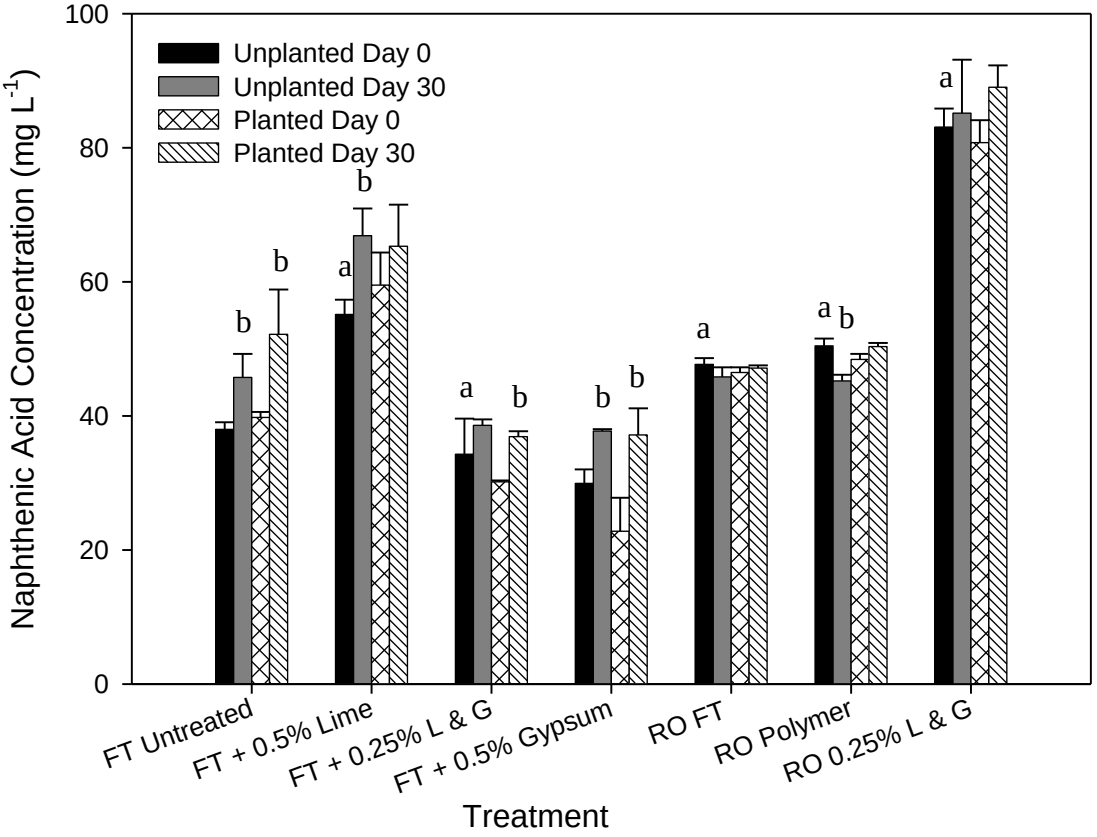

Figure 6.5: Naphthenic acid concentration (mg L^{-1}) on Day 0 vs Day 30 in different planted and unplanted tailings treatments. Values are reported as the mean ± SD (n = 3). FT = fine tailings; L = lime; G = gypsum; RO = runoff water. Significant differences between treatments and the unplanted, unamended fine tailings treatment (FT untreated) control group on Day 0 are indicated with an 'a'. Significant differences within the planted and unplanted treatments between Day 0 and Day 30 are indicated with a 'b' ($P < 0.05$).

6.4 Discussion

6.4.1 Effect of tailings treatments on plant growth

Based on the results of this study it appears that common reed growth is significantly reduced in both dilute fine tailings treatments and simulated runoff from dried fine tailings and amended fine tailings. Nevertheless, the chemical amendment of 0.5% gypsum had the lowest effect on plant growth as well as the lowest resulting NA concentration and would therefore appear to be the best treatment option of the different chemical amendments for fine tailings tested. Plants in this treatment were able to dewater the tailings at a mean rate of 22.4 mL day^{-1} ± 2.0 mL day^{-1} for plants < 1 m tall, under growth conditions (i.e. temperature and day length) similar to those observed at the oil sands operations in Fort McMurray, AB. This rate of dewatering is however, likely a minimum value in light of the fact that common reed can grow as tall as 1 – 3 m. As the runoff water from the 0.5% gypsum treatment was not tested, the effect on phytotoxicity is not established for drying and subsequent runoff water from this treatment.

The three runoff treatments were prepared from fine tailings with low absorption of added water (~ 27.7%). Consequently, high potential was anticipated for leaching of salts and other potentially toxic components in the fine tailings. This hypothesis appears to be confirmed when comparing the water chemistry data for these treatments reported in Table 6.1 (especially for concentrations of ions such as sodium, sulfur, and sulfate) and the naphthenic acid concentrations reported in Figure 6.5. Another important parameter in explaining the phytotoxicity in the 0.25% lime and gypsum treatments is the absence of carbonate ions (CO_3) in this treatment (Table 6.1). Previous research has identified that NA toxicity is inversely proportional to the concentration of carbonate (Dorn et al., 1992) Naphthenic acids form calcium naphthenate salts, thereby becoming less bioavailable and toxic (Dokholyan and Magomedov, 1983; Verbeek et al., 1994; Lu et al., 2007). The unamended fine tailings and polymer amended fine tailings runoff water contained 139 and 146 mg L^{-1} of CO_3 respectively, and the corresponding NA concentrations are ~35 and 33% lower than the 0.25% lime and gypsum treatment

containing no carbonate. Ultimately, however, NAs are only a part of the picture when consideration the phytotoxicity of OSPW. For phytoremediation, the optimum pH for plant growth must also be achieved in OSPW and runoff from dried fine tailings.

In the present study the significant reduction in plant growth was observed for a worst case scenario under optimal exposure conditions. For more realistic conditions, there would be a potential to use plants to help remediate oil sand tailings waste and assist with dewatering of fine tailings. Phytoremediation has been previously identified as being best suited as a final polishing step for contaminated medium (whether it be for air, soil, or water (Arthur et al., 2005). Dilution or further amendments may be required to remove some of the background salts that likely enhance phytotoxicity, however availability of fresh water for such dilutions is a critical issue in the region (Schindler and Donahue, 2006). Likewise, the phytotoxicity of OSPW in planted systems could be reduced using a constructed or engineered wetland treatment system with substrate and conditions optimized for plant growth (e.g. nutrition, microbial inoculants, etc.) (Arthur et al., 2005). Previous studies have used hybrid treatment systems with constructed wetlands to successfully treat brackish oil field produce water (Murray-Gulde et al., 2003).

6.4.2 Effect of chemical amendments on naphthenic acid concentrations and plants

Figure 6.5 demonstrates that planted treatments containing runoff water had no significant effect on decreasing NA concentration in tailings treatments over the course of the 30 day experiments. Although not significant in all treatments, there was a slight increase in NA concentration in some of the planted tailings treatments. This is likely a result of a buildup of NAs or salinity from watering the planted treatments with runoff water (prior to plant death in the case of those treatments which were phytotoxic).

One of the reasons why NA dissipation in planted treatments was minimal was likely because under the alkaline pH conditions observed in all of the NAs are in their water-soluble ionized form. Previous research has found that NAs are more bioavailable in their non-ionized form (Dorn et al., 1992). Because oil sands naphthenic acid has an average pK_a ranging from 5-6; the pH of the tailings would have to drop to below a pH of

6 to become more bioavailable to either plant uptake or microbial degradation from microbial communities living the rhizosphere of aquatic plants.

Factors that lead to the lowering of the pH in tailings ponds would make NAs more phytotoxic in aquatic environments. Thus, chemical amendments to OSPW such as acidification may enhance the uptake of NAs into wetland plants. It is interesting to note that the chemical tailings treatment (0.25% lime and 0.25% gypsum) has a much higher NA concentration than the simulated runoff water created from polymer treated tailings and untreated oil sands tailings. This is hypothesized to be an effect of the high pH/alkaline conditions created by this treatment, which will solubilize NAs and most likely 'wash' NAs out of the tailings treatments because NAs are likely not bioavailable in their ionized form. Because of the pH of the polymer, treated tailings were so high in pH it was difficult to determine if there was any phytotoxicity of the polymer itself and this therefore warrants further study.

Except for fine tailings diluted and treated with either 0.25% lime and gypsum, or 0.5% gypsum, chemically amended tailings or simulated runoff water increased NA concentrations compared to a simulated runoff sample from unamended fine tailings. Simulated runoff water from 0.25% lime and gypsum treated fine tailings in particular had the greatest increase in NA concentration at almost a 53% increase in NA concentration over unamended diluted fine tailings on Day 0. The suspected reason for the elevated concentration of NAs in this treatment is because the added water washed NAs from 140 kg of original tailings. Unlike the polymer amended and unamended fine tailings this treatment had the lowest absorption of water and therefore more water was free to wash out NAs from the dried tailings. This treatment also had zero carbonate ions present which when present bind to NAs (Lu et al., 2007).

6.5 Conclusions

In summary, we demonstrated that although growth was reduced, the emergent macrophyte common reed was capable of growing in diluted unamended fine tailings, as well as diluted tailings amended with either 0.25% lime and gypsum or 0.5% gypsum. As a result this plant could assist in the dewatering process of OSPW. However, despite consolidating fine tailings, runoff waters from chemically amended fine tailings are high in salts, toxic ions, naphthenic acids and pH. As a result, further remediation of runoff waters from these fine tailings using plants will be not feasible. The pH of the tailings treatment had a greater impact on the phytotoxicity of the tailings than did the NA concentration of the treatment solution.

7.0 General Discussion and Conclusions

The overall objective of this research was to determine if emergent macrophytes are capable of phytoremediating oil sands NAs. To this end, several different laboratory investigations were carried out to address the sub-objectives identified under the overall objective of the thesis (Section 1.10) and to verify the hypothesis that *emergent macrophytes would enhance the dissipation of NAs from hydroponic medium through uptake, biotransformation of NAs, and eventual incorporation into plant tissue and/or through supporting rhizosphere microorganisms which enhance NA dissipation.* Section 7.1 reviews the original sub-objectives, the experiments conducted to achieve these objectives, and finally follow with the major findings and conclusions drawn from these investigations. And finally Section 7.2 lists recommendations on future areas of research that should be conducted in light of the findings of the present research.

7.1 Synthesis and Significance of Results

7.1.1 Phytotoxicity of naphthenic acids

Develop a hydroponic testing system for the evaluation of NA dissipation and phytotoxicity in emergent macrophytes. A hydroponic test system was developed using 2.5L amber glass jars. The plant health metrics of fresh weight gain and transpiration (as determined by water uptake) were identified as being the best parameters to monitor phytotoxicity in the systems over 30 days.

Assess the phytotoxicity of naphthenic acids in emergent macrophytes. Experiments with oil sands NA extract found that this mixture appeared to be less toxic to aquatic macrophytes compared to the commercially available NA mixture (ionized, pH = 7.8). These experiments highlighted the fact that caution should be taken when making predictions on the environmental fate of oil sands NAs when using commercial NAs as surrogates. This finding had previously been identified using bacteria (Scott et al., 2005)

but had yet to be reported in plants. Another set of experiments was performed with ionized NAs (pH = 7.8) and non-ionized NAs (pH 5.0) to determine if this influenced phytotoxicity and dissipation. Here it was demonstrated that NAs appear to be more phytotoxic in their non-ionized form. Although NAs are predominantly found in their ionized form in oil sands tailings pond water, the findings of this study are important to consider for long term management of the tailings ponds. In both of these studies it was discovered that there was no significant effect of plant species on NA phytotoxicity.

Experiments were conducted on OSPW and fine tailings treatments to determine how plants performed under more realistic whole effluent conditions and experimental tailings treatments designed to accelerate the consolidation of fine tailings. Here it was demonstrated that although growth was reduced, the emergent macrophyte common reed was capable of growing in diluted unamended fine tailings, as well as diluted tailings amended with either 0.25% lime and gypsum or 0.5% gypsum and that this plant could assist in the dewatering process of OSPW. However, despite consolidating fine tailings, runoff waters from chemically amended fine tailings can be high in salts, toxic ions, naphthenic acids and pH. As a result further remediation of runoff waters from these fine tailings using plants will be not feasible without pre-treatment. Ultimately it was found that the pH of the tailings treatment had a greater influence on the phytotoxicity of the tailings than did naphthenic acid concentration of the treatment solution.

Synchrotron FTIR spectroscopic analysis of plant roots exposed to NAs indicated that there may be cellular death to the epidermis (outer root surface cells) of plant roots as well as increased lignification of the stele. Since the epidermis is important in regulating the control of nutrient uptake into the plant this may be an integral part of the mechanism of action of the compounds with regards to plant phytotoxicity. Lignification of the stele may result in blockages of the xylem and phloem and lead to water and mineral stress in the plant.

Determine the role rhizosphere bacteria play in emergent macrophyte NA phytotoxicity. Early investigations into the phytotoxicity of NAs in plants identified that there was phytotoxicity in the plants with minimal NA dissipation. This raised interest into investigating the potential of indirect phytotoxic effects of NAs in wetland plants, in

particular, the effects of NAs on the rhizosphere microbial community. Naphthenic acid exposed plants were found to have an increase presence of potentially pathogenic bacteria and a decreased presence of beneficial bacteria indicating that the phytotoxicity of NAs in plants is in part likely due to the effects of NAs on the microbial community in the rhizosphere. After NA exposure there was an increased presence of some species previously identified as hydrocarbon degraders found in oil sands tailings ponds indicative that emergent wetland plants may be able to 'seed' NA degrading bacteria into constructed wetland systems.

7.1.2 Dissipation of naphthenic acids in planted systems

Determine the dissipation of NAs and toxicity reduction in systems planted with emergent macrophytes. In experiments conducted with oil sands NA extract and a commercially available NA extract the small loss of commercial NAs from spiked hydroponic system was selective and dependant on the specific NA compound. Oil sands NA were not sequestered by wetland plants like their commercial NA counterparts. Experiments with oil sands NA extract found that this mixture was less toxic to aquatic macrophytes compared to the commercially available NA mixture (ionized, pH = 7.8).

In experiments testing the ionized (pH = 7.8) vs. the non-ionized (pH = 5.0) form of NAs there was no significant difference in NA dissipation found between planted treatments and unplanted treatments. However, when looking at the ion ratios of specific carbon numbers within specific Z families, there was evidence that wetland plants are either directly or indirectly responsible for selective NA dissipation when exposed to NAs in their non-ionized form. Dissipation of NAs was not influenced by emergent macrophyte species.

In experiments with OSPW and fine tailings treatments to determine plant performance for NA dissipation in whole effluent no appreciable NA dissipation was observed.

Synchrotron FTIR spectroscopic analysis of plant roots exposed to NAs appear to show evidence of NA uptake into the stele. In particular NAs concentrations were

identified to be the highest in the phloem where they may be transported to other parts of the plant and central root parenchyma where the compounds may be biotransformed and stored.

7.1.3 Phytoremediation of naphthenic acids

Because the present study was unable to detect the dissipation of total NAs although there was evidence of selective ion dissipation, preliminary experiments were conducted to determine if this resulted in any net reduction in toxicity. The NA medium samples were collected pre- and post- phytoremediation treatment and compared to unplanted controls. Toxicity reduction was evaluated through acute, 48 hour, LC_{50} test with the freshwater invertebrate *Daphnia magna*. These experiments found that plants significantly reduced toxicity of NA treated hydroponic medium which provides evidence that this form of remediation may benefit reclamation efforts of tailings ponds in the future.

7.1.4 Analysis of naphthenic acids in plant tissue

Develop a method to analyze naphthenic acids in plant tissue. Two different investigations were carried out to determine if NAs could be detected in plant tissue. Extraction of NAs from plant tissue using pressurized liquid extraction followed with mass spectrometry was unsuccessful using the variety of extraction parameters. Synchrotron FTIR spectroscopic analysis of root cross sections appears to demonstrate what may be NA accumulation in plant roots as well as biochemical changes in plant tissue as a result of NA exposure. Identification of NA uptake is important in determining if there is potential for NA biotransformation by the plant into less bioactive, less toxic compounds. If NAs were only adsorbing to root surface there is potential that these compounds may be released into the environment once again upon the decomposition of the plant after death.

7.2 Recommendations and future research

Highlighted in the following sub-sections are some areas identified as requiring further research to further our understanding of NA phytoremediation.

7.2.1 Toxicity reduction of naphthenic acids post-phytoremediation

Preliminary investigations into the effectiveness of phytoremediation treatments demonstrated that there was a reduction of acute toxicity in NA hydroponic medium samples after 30 days of phytoremediation. However, Lai et al. (1996) observed species differences in the analysis of toxicity reduction of NAs post microbial bioremediation. Therefore, further investigations into the toxicity reduction of phytoremediation treatments on NAs should be conducted with other species to determine if the observations witnessed in the present study are true with other species.

7.2.2 Influence of arbuscular mycorrhizal fungi on naphthenic acid phytoremediation

In the present study the effects of oil sands NAs were investigated on the rhizosphere community of the emergent macrophyte, cattail. However, several studies have identified that arbuscular mycorrhizal fungi (AMF), another plant symbiot that inhabit plant roots and the surrounding rhizosphere, can infer tolerance to the plants in contaminated environments where the plant would not otherwise survive (Haselwandter and Dowen, 1996; Turnau et al., 2008). In particular there is a growing body of evidence that AMF are capable of alleviating salt stress in plants (Sharifi et al., 2007; Colla et al., 2008). This is of interest in OSPW which is high on salts and this property has been identified as being a major concern with plant growth in OSPW (Crowe et al., 2001). Ipsilantis and Sylvia (2007) have identified that AMF do exist in the wetland plant cattail (Ipsilantis and Sylvia, 2007) but were not investigated in the present study as to determine their role in plant growth under NA exposure.

7.2.3 Plant biotransformation of naphthenic acids

The present study focused on the determination of the potential for NA phytotoxicity and dissipation from a hydroponic system. Preliminary investigations with FTIR spectroscopy indicate that there is likely uptake of NAs into plant tissue and not just adhesion to the epidermis of the plant root. To determine if plant cells are capable of biotransforming NAs into benign compounds, biochemical investigations are recommended. Specifically investigations should focus on determining the ability of the enzymes most often responsible for biotransformation, cytochrome P450 enzymes, at responding to NAs (Durst, 1991). Additional plant enzymes that have been previously identified to biotransform xenobiotics include arylacylamindase, N-glucosyltransferase, glutathione S-transferase (Sandermann, 1994) should also be investigated for potential NA biotransformation.

7.2.4 Naphthenic acid dissipation in a plant cell culture

To further elucidate the mechanism of action and uptake of NAs in plant cells, studies should be carried out with NAs in plant tissue culture. Tissue cultures with plant protoplasts in the absence of cell walls will help elucidate if plant cells can take in NAs. This system will also help identify the role the rhizosphere plays in NA dissipation by comparing whole plant data with cell culture data in the absence of rhizosphere bacteria.

7.2.5 Genetic modifications to aquatic plants for remediation

Effective, industrial scale phytoremediation efforts often require engineering to optimize the biological processes responsible for contaminant remediation. One of the engineering components of phytoremediation that is receiving more attention as of late is the use of genetically modified species with systems designed to optimize the biological processes within the plant that assist in remediation (Arthur et al., 2005). In the case of organic compounds this is often achieved through enhancing enzyme production for biotransformation of the xenobiotics. Nandakumar et al. (2005) were able to standardize an *Agrobacterium*-mediated model transformation system for cattail for the specific purpose to eventually introduce candidate genes for phytoremediation. Once the exact method of NA biotransformation and mechanism of action is determined (see Section 8.2.3) development of a cattail species that enhances the appropriate enzymes could be

developed.

7.2.6 Use of biosorbents made of plant material on naphthenic acids

In the present study there was evidence that initial application of NAs resulted in phytotoxic effects. Although there was some evidence of regrowth from plant rhizome after initial plant death, there was still evidence of dissipation in these systems. In their review on phytoremediation, Arthur et al. (2005) reported that nonliving plant material can bioaccumulate contaminants. Other studies found that dead aquatic macrophytes can serve as 'biosorbents' for heavy metal removal (Miretzky, et al., 2006). This sorption was identified to occur through an ion-exchange with counterions present in the plant biomass (e.g. cellulose). With regards to the potential of dead plant material to adsorb NAs, sorption studies have been conducted with hydrophobic organic compounds and rapeseed oil bodies (Boucher et al., 2008) this could potentially work for ~ NAs in their non-ionize lipid soluble form. Biosorption has also been noted for phenolic compounds with the dead brown algae species *Sargassum muticum* (Rubín et al., 2006). In summary, despite NA phytotoxicity there could be a role that plants play in remediation of NAs in constructed wetlands in both their living and non-living state. This is also important for understanding how constructed wetlands will respond to NA exposure during the winter when large amounts of plant material die back.

7.2.7 Development of pilot scale constructed wetland and assessment of performance of naphthenic acid remediation under field conditions

The current studies were all conducted within controlled growth chamber conditions and with individual plants in each hydroponic system. In order to determine the behavior of a multi-plant phytoremediation system in a field based system capable of handling large volumes of effluent pilot wetland studies should be conducted. Many aspects of a constructed wetland need to be engineered and tested (e.g. substrate, flow, nutrients, etc.) for performance to optimize contaminant plant interaction and plant growth for NA dissipation. As well, longer term investigations into planted systems are warranted. Only 30 day (4 week) studies could be carried out in the test systems used in the present study; however, Lai et al. (1996) found that in the case of bacterial degradation of NAs in OSPW it could take longer with evidence that degradation of NAs

took longer up to 7 to 8 weeks. Finally, macrophytes have been reported to grow better in a mixed species system rather than a monoculture (McGregor et al., 2007). This observation can also be determined in larger scale pilot systems.

7.2.8 Development of root microbial inoculants for naphthenic acid analysis in plant tissue

This research identified that upon NA exposure there appeared to be a change in the rhizosphere microbial community inhabiting the plant roots. This change appeared to result in an increase in potentially pathogenic bacteria and a simultaneous decrease in beneficial bacteria to the plant. This finding could mean that the phytotoxicity of NAs witnessed in emergent macrophytes could be an indirect effect of the rhizosphere microbes. Therefore future investigations on the use of plants for phytoremediation of NAs will require further investigation into the development of root microbial inoculants that support plant health under NA exposure. In addition, the identification of NA-specific bacteria in the cattail rhizosphere warrants further study as to what role these bacteria may play in the degradation of oil sands NAs in planted systems. Many phytoremediation designs incorporate the development of root inoculants that support plant growth (Siciliano and Germida, 1997; Johnson et al., 2004). Further research should therefore be conducted to develop root inoculants that support emergent macrophyte growth under NA exposure.

7.2.9 Development of method for naphthenic acid analysis in plant tissue.

Further efforts should be made towards the development of a method to analyze NAs in plant tissue. Future analysis of tissue extracts with high resolution mass spectrometry may be able to discern the presence of NAs in plant tissue extracts where low resolution mass spectrometry used in the present study could not.

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APPENDIX A: Instrument Operating Parameters for Naphthenic Acid Analysis using Negative Ion Electrospray Ionization Mass Spectrometry

Hydroponic medium samples were analyzed for NAs using a Quattro Ultima triple quadrupole mass spectrometer (Waters Corp. Milford, MA, USA) equipped with an electrospray ionization (ESI). The instrument operating parameters used in this thesis for analysis of naphthenic acids using negative ion electrospray ionization mass spectrometry are reported in McMartin et al. (2004) as well as listed in Table A1. A 2695 Alliance Separation Module (Waters Corp. Milford, MA, USA) was used for solvent delivery and sample introduction. Loop injection was employed with a sample volume injected of 5µL. Eluent used was 50:50 acetonitrile in water with 0.1% ammonium hydroxide at a flow rate of 200µL/min. Masslynx 4.1 (Waters Corp. Milford, MA, USA) was the instrument software used for data collection and processing.

Table A.1: Instrument operating conditions for the Quattro Ultima triple quadrupole mass spectrometer (Waters Corp. Milford, MA, USA) equipped with negative ion electrospray ionization (ESI).

	Parameter Description	Parameter Setting
1	Source Temperature	90 ^{o}C
2	Capillary Voltage	2.63 kV
3	Cone Voltage	62 V
4	Cone Gas (N_2)	158 L h^{-1}
5	Desolvation Temperature	220 ^{o}C
6	Desolvation Gas (N_2)	489 L h^{-1}
7	Low Resolution	14.1
8	High Resolution	14.3
9	Ion Energy	1.7
10	Entrance Voltage	95 V
11	Exit Voltage	55 V
12	Multiplier	450 V

APPENDIX B: Preliminary Data on Analytical Interference by Hydroponic Medium and Effectiveness of Solid Phase Extraction Clean Up Procedure

Preliminary analysis of naphthenic acids in hydroponic medium found that there was a high concentration of NAs in the hydroponic medium from the planted control systems where no NAs had been added (Figure B.1). This indicated that the nutrient salts in the medium were causing interference in the analytical analysis of NAs. A solid phase extraction clean-up step (Section 2.3.4) was introduced to remove the salts from the medium which reduced the signal in the controls (Figure B.1).

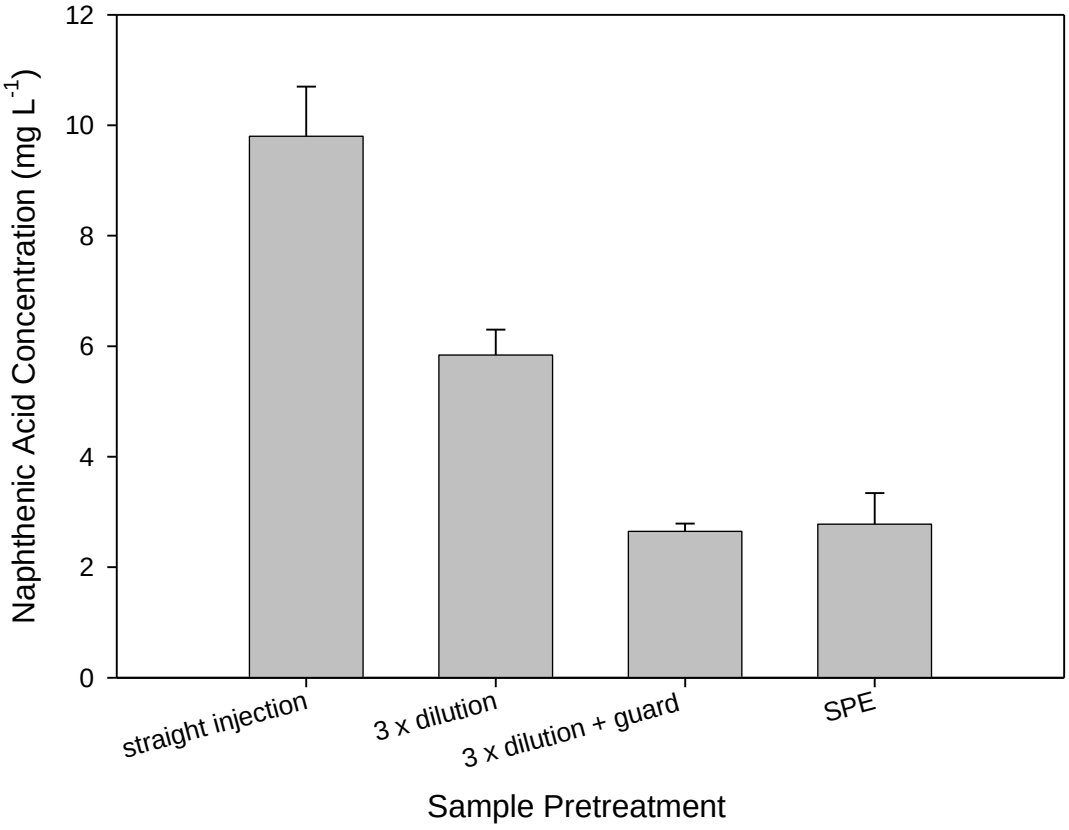

Figure B.1: Naphthenic acid concentration (mg L^{-1}) in control hydroponic medium (0 mg L^{-1} naphthenic acids) on Day 0 (n = 3. mean ± SE). Strait injection yields a high signal likely from the medium salts. Diluting sample three times (3 x dilution), three times dilution plus a guard column (3 x dilution + guard), and solid phase extraction with ENV+ cartridge (SPE).

Three times dilution plus followed with a guard column appeared to be as effective at reducing the background signal from the hydroponic medium (Figure B1) however the guard column influenced the analysis of samples with naphthenic acids compared to the solid phase extraction clean up method.

APPENDIX C: Evidence of Plant Re-growth after Die-off after Oil Sands Process Water Exposure

Despite the phytotoxicity observed in plants hydroponically exposed to non-ionized NAs, some of the planted treatments were able to recover by sending out new shoots from the rhizome (Figure C.1). This re-growth of plant tissue after contaminant exposure and subsequent dieback has been reported previously (Delaune et al., 2003). Here, the authors reported a greater recovery from oiling by crude oil in the field sites versus plants in a greenhouse study in a variety of wetland plant species (including cattail). Delaune et al. (2003) attributed the regeneration in field studies to be a result of having a more extensive root system in the field and because the roots are major storage sites for carbohydrates and thus the plant could recover.

Figure C.1: Re-growth of common reed from roots after exposure and subsequent phytotoxicity to main shoot.

APPENDIX D: Algal Growth in Oil Sands Process Water and Naphthenic Acids

To further elucidate the uptake and mechanism of action and uptake of NAs in plant cells a simpler plant cell system must be investigated. One such system involves working with tissue cultures with plant protoplasts in the absence of cell walls will help elucidate if plant cells can take in NAs. This system will also help identify the role the rhizosphere plays in NA dissipation by comparing whole plant data with cell culture data in the absence of rhizosphere bacteria. In addition to working with such a system single cell algae are also considered to be a model system for relating NA uptake and effects to higher plants. In addition to modeling single cell response to NA exposure, freshwater algae are an important component of wetland systems. Therefore studying the behaviour in OSPW remediation scenario warrants investigation.

Although the present research found reduced growth upon exposure to OSPW and NAs in macrophytes, Nix and Martin (1992) found that OSPW high in NAs caused enhanced growth of the fresh water green algae. The following Appendix outlines preliminary experiments conducted with the standard freshwater green algae *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*) in OSPW, NAs, and fine tailings treatments tested in the previously mentioned research with emergent macrophytes (cattail, common reed, bulrush).

Materials and Methods

The freshwater algae *Pseudokirchneriella subcapitata* (UTCC 37) was obtained from the University of Toronto Culture Collection and cultured under the conditions outlined in the Environment Canada biological testing method for growth inhibition tests using the freshwater alga (Environment Canada, 2007). The tests were carried out in 50 mL erlenmeyer flasks with 10 mL of treatment medium. The different treatments tested are outlined below. After the 72 hour growth period the algae were counted using a hemocytometer. Treatment 1 is the control, Treatment 2-5 are of OSPW, Treatment 6-8 are simulated runoff water from fine tailings investigated in Section 6, and finally

treatment 9 – 11 is of naphthenic acids only (Table D.1). Each treatment was replicated three times (n = 3).

Analysis of NAs was achieved by transferring the treatment solution (10mL) from the erlenmeyer flask into a centrifuge tube. The treatments were then centrifuged at 2000 G for 15 minutes. After centrifuging a ~ 2mL sample was transferred to an LC vial and then was cleaned up further using a previously reported SPE method (Armstrong et al., 2008). The analytical analysis was carried out using ESI-MS and is reported in Armstrong et al., 2008).

Table D.1: Name and descriptions of treatments used in algae experiments with oil sands process water fine tailings and naphthenic acids.

	Treatment	Treatment Information
1	Control	Algae growth medium
2	25% OSPW	25% oil sands process water + 75% water
3	50% OSPW	50% oil sands process water + 50% water
4	75% OSPW	75% oil sands process water + 25% water
5	100% OSPW	100% oil sands process water
6	FT	Simulated run-off water from fine tailings (FT)
7	FT + Polymer	Simulated run-off water from FT + polymer amendment
8	FT + Gypsum	Simulated run-off water from FT + 0.25% lime & gypsum
9	NA60 + 10xA	60 mg/L naphthenic acids + 10^5 cells/mL algae
10	NA60	60 mg/L naphthenic acids
11	NA30	30 mg/L naphthenic acids

Results and Discussion

Although growth was significantly reduced ($P < 0.05$), algae were found to grow in undiluted OSPW (Figure D.1). With a 75% dilution there was a 32.6% reduction in growth compared to controls and at a 50% dilution of OSPW this reduction in growth increased to 71.5%. Simulated run-off water from mature fine tailings and chemically amended mature fine tailings were acutely toxic to algae (Figure D.1). These findings are in contrast to what was observed by Nix and Martin (1992) who reported that OSPW high in NAs caused enhanced growth of the fresh water green algae.

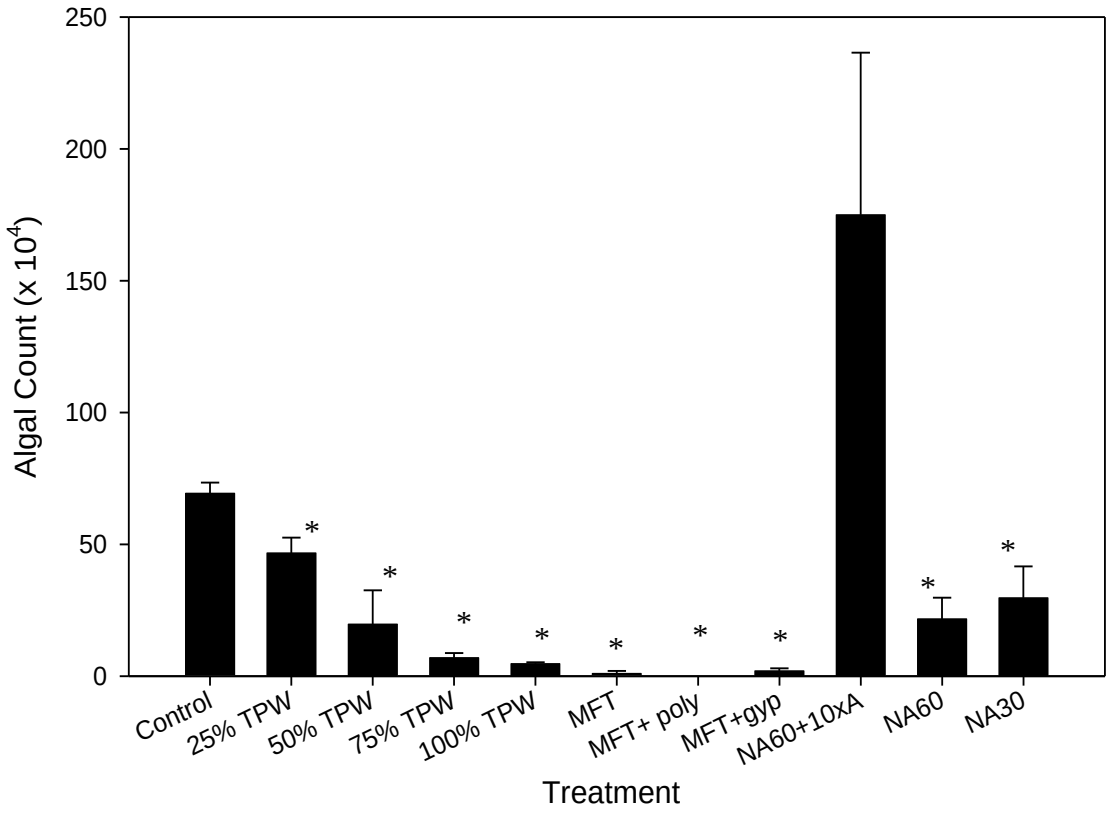

Figure D.1: Algal counts (x 10^4) for the freshwater algae *Pseudokirchneriella subcapitata* with a starting concentration of 10^4 cells per mL after 72 h in different treatments. Algal counts are reported as the mean ± SD (n = 3) and significant differences from the control are indicated with an asterisk ($P < 0.05$). Note treatment NA60 + 10xA started with 10^5 cells mL^{-1} to determine the influence of the amount of algae on naphthenic acid concentration and was not compared to the control.

Figure D.2 plots the correlation between starting NA concentration and the algal counts in treatments after 72 hours. There is a definite negative relationship noted and at concentrations of 40 mg L^{-1} or greater algae growth reduces to zero (Figure D.1). This trend has been notice in macrophytes (Section 6.0) and the concentration of NAs that results in zero fresh weight gain is approximately the same (40 mg L^{-1}) (Figure 6.3).

Even with sample pre-clean up, the presence of algae in the medium interfered with the analysis of NAs in the medium. This is evident when looking at the NA concentrations by 72 h in Figure A.3 which are higher than the starting concentration. Despite this; however, it is interesting to note that the treatment with 10x the starting algae concentration has significantly lower NA concentration than the normal algae treatment (10^4 cells mL^{-1}) indicating potential NA dissipation. Although not attempted in the present study because of concerns over loss of analyte, future studies should attempt pre-filtering the medium to separate the algae prior to analysis (0.45 μm).

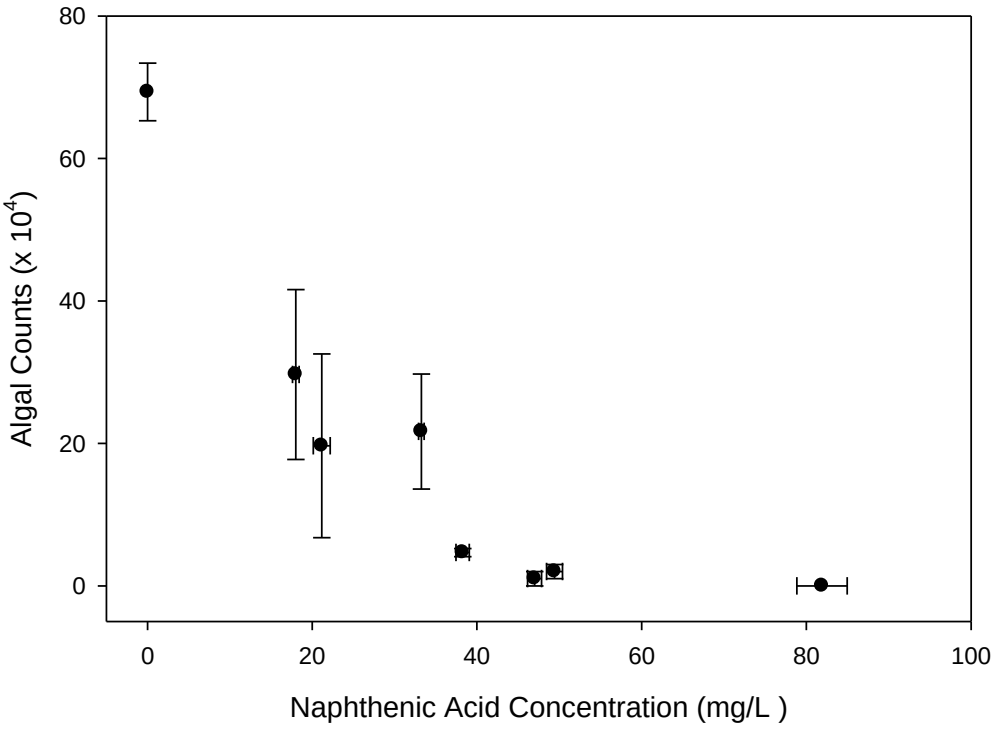

Figure D.2: Algal counts (x 10^4) for the freshwater algae *Pseudokirchneriella subcapitata* with a starting concentration of 10^4 cells per mL after 72 h in different treatments plotted against the naphthenic acid concentration (mg L^{-1}) of the treatment. Algal counts and naphthenic acid concentrations are reported as the mean ± SD (n = 3).

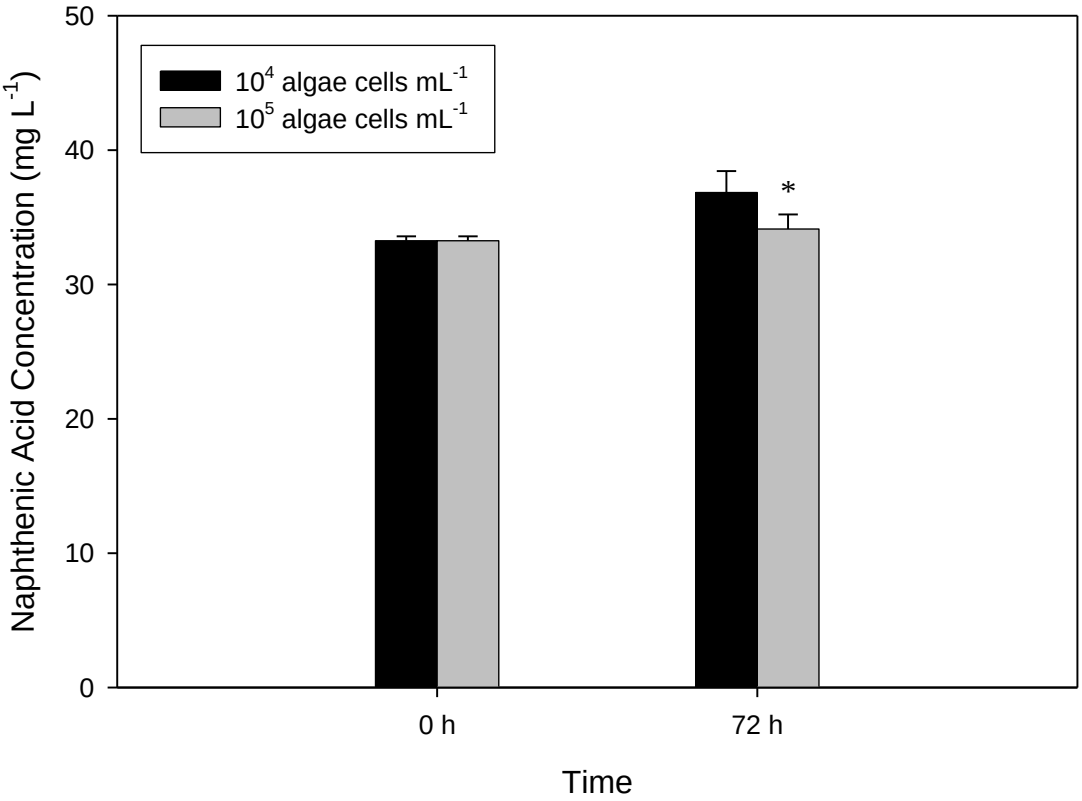

Figure D.3: Naphthenic acid concentration of medium (mg L^{-1}) spiked with 60 mg L^{-1} after 72 h incubation with algae at a starting concentration of 10^4 algae cells mL^{-1} and 10^5 algae cells mL^{-1}. Naphthenic acid concentration is reported as the mean ± SD (n = 3). Significant difference between treatments reported with an asterisk (* ; $P < 0.05$).

Conclusions

In conclusion dilution of OSPW was found to increase the potential for phytoremediation with algae. Run-off water from dried fine tailings and fine tailings amendments were found to be more toxic than the original OSPW. Naphthenic acid toxicity appeared to be similar to that observed in aquatic macrophytes therefore the use of freshwater algae for NA dissipation studies and investigations on NA mechanistic studies may be possible. Finally significantly lower NA concentration in algae treatments with 10x more starting algae concentrations indicates that NAs may be taking in NAs thereby enhancing dissipation.